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APPROVAL SHEET

Title of Dissertation: "Mechanisms for Development and Function of Foxp3+ Regulatory T Cells"

Name of Candidate: Cristina Stoica
Doctor of Philosophy Degree
4 April 2008

Dissertation and Abstract Approved:

Gabriela Dveksler, Ph.D.
Department of Pathology
Committee Chairperson — 4/14/08 Date

Teodor Brumeanu, M.D.
Department of Medicine
Committee Member — 9/5/08 Date

Charles Via, Ph.D.
Department of Pathology
Committee Member — 04/04/2008 Date

Stephen Davies, Ph.D.
Department of Microbiology & Immunology
Committee Member — 04/04/2008 Date

Andre Dubois, M.D., Ph.D.
Department of Medicine
Committee Member — 04/04/08 Date

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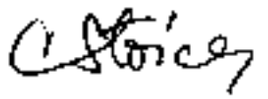


Cristina M. Stoica
Molecular and Cell Biology Program
Uniformed Services University of the Health Sciences

ABSTRACT

Title of Dissertation: "**Mechanisms for Development and Function of Foxp3+ Regulatory T Cells"**

Author: **CRISTINA MIRELA STOICA**, PhD, 2008

Uniformed Services University of the Health Sciences,
4301 Jones Bridge Rd., Bethesda, MD 20814

Thesis directed by: **Teodor – D. Brumeanu, MD.** Associate Professor,
Dept. of Medicine and Dept. of Molecular and Cell Biology.
Uniformed Services University of the Health Sciences
4301 Jones Bridge Rd., Bethesda, MD 20814

Regulatory T cell – mediated dominant tolerance has been demonstrated to play an important role in the prevention of autoimmunity. In this dissertation we present evidence that the marker gene of T reg cells (Foxp3) is expressed in a very early stage of thymic differentiation. By studying two different murine genetic backgrounds, we found several mechanisms leading to a differential thymic T reg development and function. While high rates of proliferation occurred in T-reg precursors with low Leptin receptor density, high rates of apoptosis occurred in T-reg precursors with elevated Bad, Caspase 3, and CD3 expression. Despite a differential T-reg thymic output, the size of peripheral $CD4^{+}25^{hi}Foxp3^{+}$ T-reg subset was normalized regardless the genetic background. Conversely, the T-reg

suppressogenicity as tested in a model of autoimmune diabetes was directly correlated with the level of Foxp3 expression. A second important finding is that CD28 costimulation alone augmented, but did not induce de novo Foxp3 expression in T-reg thymic precursors. The CD28-upregulation of Foxp3 relied on mRNA stabilization, and required plasma membrane rafts integrity as well as a functional $p56^{lck}$ binding motif on the CD28 cytosolic tail. Noteworthy, the glycosphingolipids and cholesterol rafts components were unusual partitioned in T-reg precursors as compared with conventional T-cell precursors. The CD28-upregulation of Foxp3 was paralleled by an increase in thymic proliferation and in suppressogenicity of terminally differentiated $CD4^{+}25^{hi}$ T-reg thymocytes. Extending the life span of Foxp3 transcripts in a rafts/$p56^{lck}$ manner represents a novel mechanism by which the CD28 co-receptor fosters the development and suppressogenicity of $CD4^{+}25^{hi}$ T-reg precursors in the thymus.

Mechanisms for Development and Function of Foxp3+ Regulatory T Cells

by

Cristina Mirela Stoica

Dissertation submitted to the Faculty of the Department of Molecular and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 2008

Acknowledgements

I would like to express my gratitude to the following people:

- . Dr. Teodor Brumeanu, for being a good mentor, for all the discussions and productive brainstorming meetings we had, for guiding me through several projects and allowing me to participate in national conferences.
- Dr. Constantin Bona, for teaching me what scientific research really means, and for being a great scholar and mentor.
- Drs. Gabriela Dveksler, Stephen Davies, Andre Dubois and Charles Via, for their helpful suggestions and positive feedback, and for being extremely open to all the ideas I presented to them.
- colleagues at USUHS: Anca Preda, Anthony Foster, Beverly Duncan, Kateryna Lund, Karen Wolcott.
- my family back in Romania, and my future parents in law here, for their constant support and for believing in me.
- last, but definitely not least, my fiancé Ilya Nazarov, for being patient, wise and very, very supportive.

List of Abbreviations

APC: (antigen presenting cell): a cell that displays foreign antigen complexed with MHC on its surface

APC: allophycocyanin fluorochrome excited at 600 nm and emits at 660 nm

BCL-2: B-cell lymphoma 2, pro-apoptotic gene

CD3: a critical signaling component of the T cell receptor complex

CD4: a co-receptor for the T cell receptor expressed on the surface of T helper cells

CD8: a co-receptor for the T cell receptor expressed on the surface of cytotoxic T cells

CD25: the α chain of the high-affinity interleukin-2 receptor

CD28: T cell-surface antigen that provides co-stimulatory signals required for naïve T cell activation

CD103: alpha E integrin

CDK4: cyclin dependent kinase

cDNA (complementary DNA): DNA synthesized from an RNA template

CFSE: Carboxy Fluoroscein Succinimidyl Ester

cMAF: T helper (Th)2 cell-specific transcription factor

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

CTX-B: Cholera Toxin B subunit

DN: double negative T cells. They do not express CD4 or CD8 on their surface

DP: double positive T cells. They express both CD4 and CD8 on their surface

DRB: 5,6-dichloro-1-β-D-ribofuranosyl-benzimide-azole

FACS (fluorescence-activated cell sorting): methodology that uses lasers and microfluidic cell separation to identify unique cell types based upon differences in cell fluorescence

Fas: cell surface receptor protein of the TNF receptor family known also as CD95, that induces apoptosis on binding Fas ligand

FITC: fluorescein isothiocyanate; a fluorochrome which excites at 488nm and emits at 520nm.

Foxp3: forkhead box protein 3. Marker of T reg cells

GATA3: transcription factor characterized by its ability to bind to GATA sequence

GITR: glucocorticoid-induced tumor necrosis factor receptor family-related gene

H & E: hematoxylin and eosin staining.

HA: hemagglutinin protein from influenza virus

Ig: Immunoglobulin; Ab

IL-2 (interleukin-2): an autocrine cytokine secreted by T cells that stimulates T cell growth and differentiation

IPEX: Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked

KO: knock out

MβCD: Methyl-β-cyclodextrin

MFI: Mean Fluorescence Intensity

MHC (major histocompatibility complex): genomic region that encodes for cell-surface molecules that present peptide antigen to the T cell receptor

NFAT: Nuclear factor of activated T-cells

ObR: Ob receptor, leptin receptor

PE: phycoerythrin fluorochrome, which is excited at 488nm and emits at 580nm.

PerCP: Peridinin chlorophyll protein – fluorochrome excited at 490nm and emits at 675nm

RAG: recombination-activating protein

RT-PCR: reverse transcriptase polymerase chain reaction

SFN: scurfin protein

STAT: Signal Transducers and Activator of Transcription

TCR (T cell receptor): cell-surface receptor of T cells that binds a peptide of processed cognate antigen complexed with MHC

TcH: T-cell Hybridoma

Th1: T helper type 1 cell; producers of IFN-γ and IL-2, and are important in anti-bacterial, viral, and cancer responses.

Th2: T helper type 2 cell; producers of IL-4, IL-13, and IL-5, and are involved in anti-helmith and allergic responses.

TLR: toll like receptor; a family of innate PAMP receptors

TNF: Tumor necrosis factor

T reg: regulatory T cell

WT: wild type

Table of Contents

List of Additional Figures:

DISSERTATION INTRODUCTION:

The ability to discriminate between self and nonself is perhaps the most fundamental aspect of immune regulation. This property translates into the immune recognition and may serve for destruction of infectious agents, whilst normal tissues are not affected. The immune system has extensive mechanisms for preventing the attack of healthy self tissues by various infections and chemical agents. The first and major step of 'self-tolerance' is the elimination of self-reactive T lymphocytes and B lymphocytes during negative selection in the thymus and bone marrow, respectively (central tolerance). However, this mechanism is not perfect, and autoreactive clones may escape into the periphery. There has long been a belief that the immune system must have peripheral mechanisms in place to deal with immune cells that 'escape' central tolerance. For almost 40 years, immunologists have postulated the existence of suppressor T cells that help the immune system to avert unwanted immune responses (Benacerraf et al., 1975, Bach et al., 1990). Recently, the identification of a set of cell surface, transcriptional and biochemical markers that uniquely mark 'regulatory' T cells (Treg cells) has made possible the rebirth of the suppressor T cell field over the past decade. It has been proven that Treg cells have the ability to inhibit the development of autoimmunity when transferred into the appropriate host.

Treg cells have a unique surface expression profile of protein markers including CD25, CD62L, specific CD45 isoforms (Sakaguchi et al., 1995, Herbelin et al., 1998, Hall et al., 1990), and several other surface receptors such as CTLA-4, OX40 (TNFRSF4), glucocorticoid induced TNF receptor super family member (GITR), TNFR2,

4-1BB, PD1, CD103 (αEβ7 integrin), Toll Like Receptors (TLR), and the recently described galectin-10. (Caramalho et al, 2003, McHugh et al, 2002, Kubach et al, 2007, De Rosa et al, 2007, Read et al, 2006). A deeper understanding of Treg biology was achieved with the discovery of Treg cell–specific *forkhead*/winged-helix transcription factor Foxp3, which was shown to be expressed predominantly in Tregs and to be both necessary and sufficient for their development and function (Hori et al, 2003). All these markers catapulted Treg cells from a rare CD4+ T cell subset to what many regard as 'master regulators' of immune homeostasis (Fontenot et al, 2003, Khattri et al, 2003).

Recently, it has become evident that there is a myriad of T reg cell subpopulations, including the CD4+CD25+ Foxp3+ cells, interleukin 10 (IL-10)–producing 'Tr1' cells (Roncarolo et al, 2006), transforming growth factor-β (TGF-β)–producing T helper type 3 cells (Faria et al, 2005), CD8+ T suppressor cells (Chang et al, 2002), natural killer T cells (Kronenberg et al, 2005), CD4–CD8–T cells (Zhang et al, 2000) and γδ T cells (Hayday et al, 2003). Most of these Treg cells, such as the CD4+Foxp3+ cells, originate in the thymus during ontogeny and are referred to as 'natural' Treg cells. As an important piece of evidence, it has been shown that very early neonatal thymectomy (before day 3 after birth) associates with spontaneous development of autoimmune diseases including gastritis, thyroiditis, oophoritis in different strains of mice (Sakaguchi et al, 1982, Sakaguchi et al, 1985, Asano et al, 1995). However, T reg cells can also be induced from naive T cells in the periphery (Bluestone and Abbas, 2003). Some, but not all of these peripherally induced 'adaptive' Treg cells also express Foxp3.

The Foxp3+ CD4+ natural Treg cells, which develop in the thymus, are central for immune homeostasis, as illustrated by the fatal consequence of their absence from

mice deficient in IL-2, CD25 or Foxp3, or of their depletion from normal adult mice (Malek et al, 2002, Kim et al, 2007). An important finding showed that retroviral transduction of Foxp3 into Foxp3- nonregulatory, peripheral CD4+25- T cells led to a fully functional T reg phenotype (Fontenot et al, 2003). Notably, like mice, humans with mutations in *FOXP3* develop multiorgan autoimmune diseases with fatal consequences – immune dysregulation poly-endocrinopathy enteropathy, X-linked syndrome (IPEX) - (Gambineri et al, 2003). Thus Foxp3 is a master regulatory gene for the development and function of CD4+25+ Treg cells (Figure I).

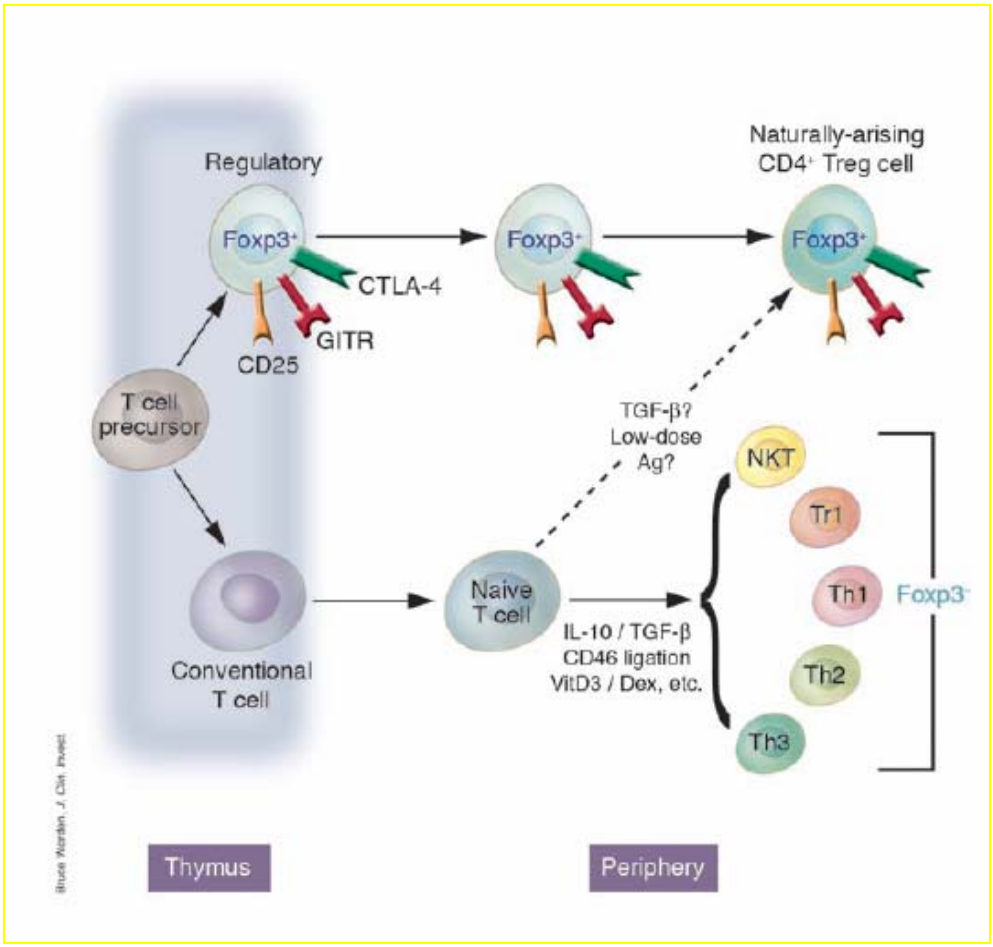


Figure I: Mechanisms for the development of Treg cells.

Several reports addressed the ontogeny of T reg cells, as it was shown that in the fetal thymus CD4+25+ Treg cells display surface features similar to those reported in post-natal thymocytes: expression of GITR, CD122, intracellular CTLA-4, low expression of CD127. Notably, CD4+25+ Treg fetal thymocytes have the potential to suppress proliferative responses of autologous CD4+25- T cells as effective as mature T reg cells, (Cupedo et al, 2005, and our unpublished data).

Previous reports have suggested that CD4+25+ thymocytes might be educated during the selection steps to recognize self-antigens that are presented on MHC class II molecules by stromal cells in a process that is known as "altered negative selection", after which they migrate directly to peripheral lymphoid tissues (Jordan et al, 2001, Bensinger et al, 2001, van Santen et al, 2004).

Although CD4+25+ Treg cells express an αβ T cell receptor (TCR) and can be found in the thymus as well as in the peripheral lymphoid organs, their ontogeny has been a matter of continuous debate. It is known that Treg cells are produced in the thymus and that the thymic and peripheral CD4+25+ Treg cells share lineage continuity (Itoh et al, 1999). There is also evidence that agonist ligands can achieve the conversion of naïve T cells into suppressor CD4+25+ Treg cells (Apostolou et al, 2004, Mahnke et al, 2003, Thorstenson et al, 2001).

Thymocyte differentiation progresses from early CD4-CD8- (double negative) progenitors either directly to TCR-γδ+ cells, or going through several steps of differentiation like CD4+CD8+ (double positive) cells, which are then selected by TCR – peptide engagement into mature "single positive" CD4+ and CD8+ TCR-αβ+ cells. The

double negative to double positive transition step depends on a pre-TCR comprising TCR-β and pre-Tα chains. Treg cell development is suggested to be determined by high-affinity TCR - peptide agonist engagement of double-positive cells (Kawahata et al, 2002, Jordan et al, 2001, Stephens et al, 2003). It appears that thymocytes expressing relatively high-affinity TCRs following receptor editing commit to a Treg developmental program involving activation of Foxp3 gene. An intermediate level of signaling would result in the generation of conventional effector T cells (Figure II), therefore generation of Treg cells require a balance between deletion and progression towards effector T cells. The normal thymus is continuously producing pathogenic self-reactive CD4+ T cells as well as CD4+25+ Treg cells capable of controlling them. This centralized production of Treg cells has been referred to as "the third function of the thymus" (Seddon and Mason, 2000).

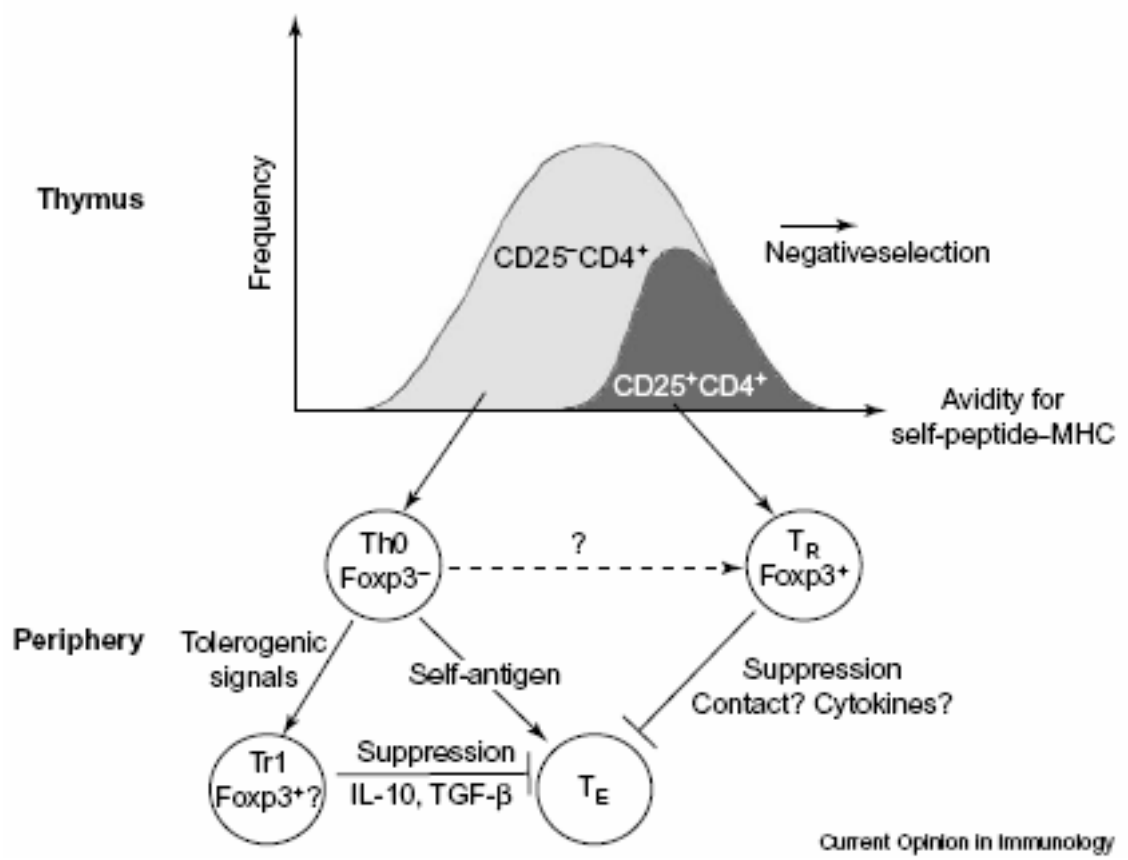
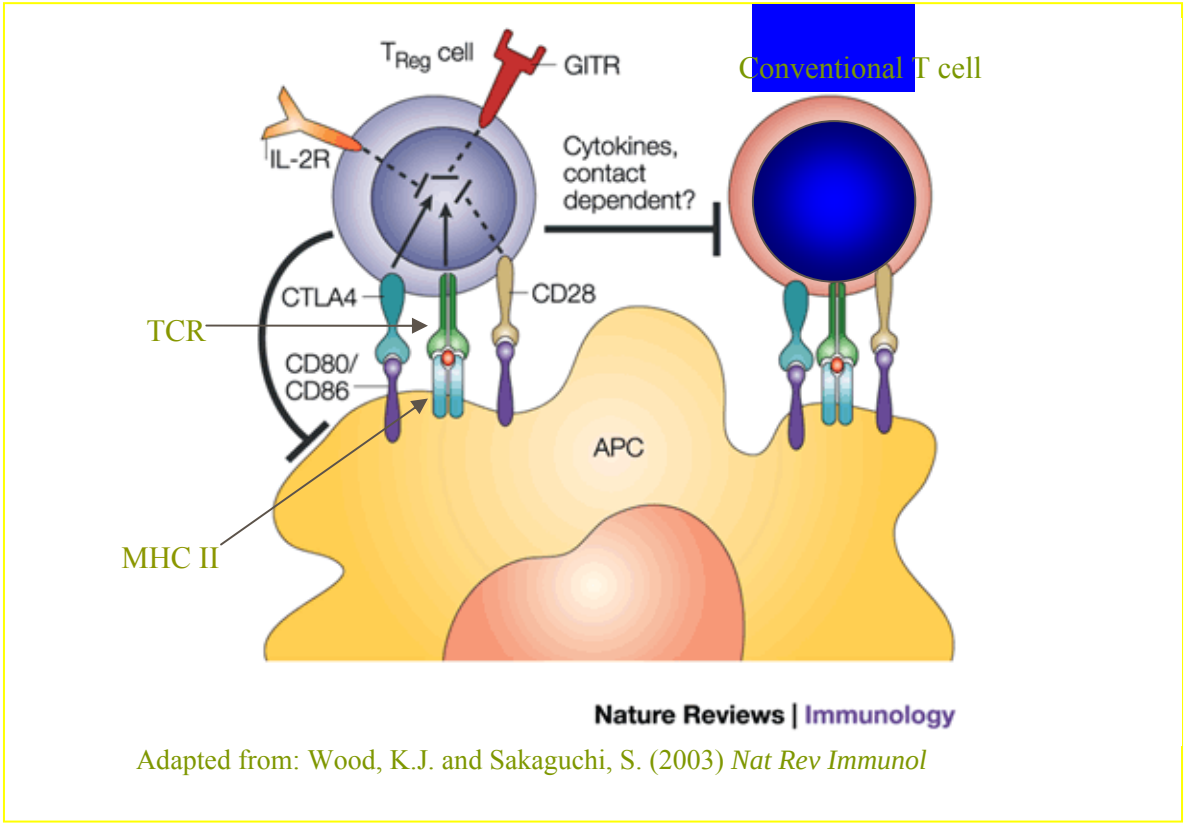


Figure II: Treg-cell development and maintenance of self-tolerance. The T-cell repertoire is shaped in the thymus by the processes of positive and negative selection. Avidity determines the fate of the T cell; too low and its fails to receive necessary survival signals, too high and the potentially auto-reactive cell is actively deleted. Cells of intermediate avidity remain Foxp3_ and differentiate into conventional effector cells (Th0). Relatively self-reactive T cells (but not so much as to be deleted) activate a Foxp3 developmental program and are exported as TR cells. Autoreactive Th0 and TR cells respond to antigenic stimulation by either differentiating into effector T cells ('TE'; Th1/Th2), or engaging in active suppression, respectively. Additionally, Th0 cells can differentiate into regulatory cells under tolerogenic conditions in the periphery (Tr1) then mediate suppression through the production of immunomodulatory cytokines.

The fine mechanisms of Treg cells development in thymus and periphery remain under scrutiny, as it is still unclear if they represent a specific cell lineage or not. A cell specific lineage refers mostly to a common unique stem cell precursor, and less to a differentiation process of a particular cell population. Delineating the processes underlying Treg cell development will be essential for their therapeutic application.

Immuno regulation is a main feature of Treg cells, since several reports showed that the transfer of Treg cells into animals developing autoimmune diseases prevented the progression of the disease (Boitard et al, 1989, Powrie et al, 1990, Sakaguchi, 2004). Although important progress has been made in deciphering the role of Treg cells in the immune system, the exact mechanisms of suppression remain elusive. There is general consensus is that they act in a cell-to-cell contact manner, in which a possible mechanism

may rely on deprivation of autoreactive T cells of exogenous IL-2 generated by T responder cells through an autocrine mechanism (de la Rosa et al, 2004). Another mechanism of T reg suppression discovered recently involved CTLA-4 triggering the induction of indoleamine 2,3-dioxygenase (IDO) by dendritic cells, which catalyzes the tryptophan conversion into kinurenins, molecules with immunosuppressive effects on different types of cells (Fallarino et al, 2003).



Adapted from: Wood, K.J. and Sakaguchi, S. (2003) *Nat Rev Immunol*

Figure III: Mechanism of action of Treg cells.

One mechanism designed to maintain the fidelity of the immune response is the requirement of two distinct signals for effective activation of antigen-specific T cells: an antigen-specific signal via the T cell receptor (Signal 1) and a non-antigen specific (noncognate) costimulatory signal (Signal 2) that is provided by cell-surface molecules on the antigen presenting cell (APC). The identification of a costimulatory signal has important implications for clinical intervention as the effects of costimulation blockade would be restricted to only those T cells whose antigen-specific receptors have been engaged, i.e. T cells already receiving signal 1. Although investigators have studied the T cell receptor and its fundamental function for decades, they only recently discovered the existence of the second costimulatory signal. Experiments in the late 1980s showed that T cell clones failed to proliferate in the absence of costimulatory signals and became refractory to further activation (Jenkins and Schwartz, 1987). The finding that T cell inactivation, termed T cell anergy, was a direct consequence of lack of IL-2 production (DeSilva et al, 1991), which led to the search for a master costimulatory signal that targeted the IL-2 pathway. This search resulted in the identification of the CD28/B7 pathway as the archetype costimulatory pathway for T cells (Harding et al, 1992, Jenkins et al, 1991). Although additional costimulatory pathways have since been identified, including CD40 ligand (CD154)/CD40, CD2/CD58, LFA-1 (CD18)/ICAM-1 (CD54), and several others, the CD28/B7 pathway remains one of the most potent and well-characterized costimulatory interactions. There is increasing evidence that CD28 engagement leads to multiple effects on immune responses in addition to the regulation of IL-2 production. The pleiotropic activities of CD28 support the potential clinical usefulness of CD28/B7 blockade in immune intervention. The effectiveness of

costimulatory blockade was first demonstrated in the early 1990s using CTLA-4Ig, an engineered fusion protein that binds with high affinity the two ligands of CD28, namely B7-1 (CD80) and B7-2 (CD86), expressed by APCs (Linsley et al, 1992). This antagonist interaction inhibited islet xenograft rejection and induced long-lasting immune tolerance (Lenschow et al, 1992). Subsequently, a number of preclinical studies have demonstrated the primary importance of the CD28/B7 pathway in the prevention of graft rejection in rodents and primates. Antagonists of this pathway, alone or in combination with CD40L/CD40 blockade, have been especially effective in treating autoimmune diseases in preclinical models of these diseases (Levisetti et al, 1997, Kirk et al, 1997, Elwood et al, 1998). However, the complexity of regulation of these diseases by T cell costimulation blockade has now become evident.

Lipid rafts are the detergent-insoluble fraction of the plasma membrane, and they represent a platform for the very early signaling events in T-cells (Simons and Ikonen, 1997). Rafts are actively involved in the assembly of immunological synapse between APCs and T cells. They aggregate at the site of TCR engagement and act as foci for triggering the intracellular signaling machinery (Montixi et al, 1998).

Several findings argue in favor of a role for lipis rafts in T cell function: plasma membrane GM gangliosides that are permanent residents of lipid rafts, and cholesterol, are differentially partitioned in the neonatal, adult, and senescent CD4 T-cells, as well as in the thymic precursors *vs.* their mature CD4 T-cell counterparts (studies by our group - Brumeanu et al, 2007); GM gangliosides interact physically with TCR and CD4 in the lipid rafts of quiescent and activated CD4 T-cells increasing the stability of the immunological synapse (studies by our group - Thomas et al, 2003); Cholesterol

synthesis and thereby the cholesterol amount in CD4 T-cells can be modulated in vitro and in vivo. It can be either reduced by HMG CoA reductase inhibitors, or increased by exogenous addition of squalene, a precursor of cholesterol synthesis (studies by our group - Brumeanu et al, 2007).

At present, it is generally accepted that lipid rafts play a critical role in the conventional T cells, but their role in T reg function remains still unknown.

The studies presented in here show first, that the $CD4^{+}25^{hi}Foxp3^{+}$ T-reg precursors followed different patterns of thymic differentiation in two mouse strains of different genetic backgrounds, BALB/c and B10D2, but sharing the same H-2^{d} MHC class II haplotype. Differential development of $CD4^{+}25^{hi}Foxp3^{+}$ T-regs in these mouse strains led to variations in the thymic output and threshold of suppressogenicity of the mature $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg pool. A dual mechanism responsible for such variations relied on the rates of proliferation and apoptosis in T-reg precursors, whilst the suppressogenic thresholds of mature $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg cells in periphery was much related to the level of Foxp3 expression.

Second, we provide evidence for a new mechanism by which CD28 costimulation solely up-regulates the Foxp3 expression in T-reg thymic precursors through stabilization of Foxp3 mRNA transcript. The $p56^{lck}$ binding motif on the cytosolic CD28 tail, but not the CD28-mediated PI-3K activation was critical for stabilization of Foxp3 mRNA transcript. We also show that the CD28 costimulation alone fosters the cell cycle division of T-reg precursors in thymus, and enhances the suppressogenic capacity of terminally differentiated $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg thymocytes. These signaling events were strictly depending on the integrity of plasma membrane lipid rafts on T-reg precursors.

MANUSCRIPT 1

Genetic Imprints on the Development and Function of $CD4^{+}25^{hi}$ T Regulatory cells

Cristina Stoica[1], Constantin Bona[2], Sofia Casares[3], and Teodor-D. Brumeanu *[1]

[1] Department of Medicine, Division of Immunology, Uniformed Services University of Health Sciences, Bethesda, MD 20814, U.S.A;

[2] Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, U.S.A;

[3] Naval Medical Research Center, Infectious Diseases Directorate, Malaria Program, Silver Spring, MD 20910, U.S.A.

* Correspondence: tbrumeanu@usuhs.mil

Keywords: Foxp3, T regulatory cells, Thymic development, Suppressogenicity, Genetic background.

Running title: Development and function of T-reg cells

SUMMARY

We provide evidence that Foxp3 expression, the master-regulator gene for T-regulatory cells (T-reg), occurs in early stage of thymic differentiation, particularly in the $CD4^{-}8^{-}$ DN3/4 thymocytes. A dual mechanism leading to various patterns of thymic development and function of T-reg cells was revealed in two different murine genetic backgrounds sharing the same MHC II haplotype While high rates of proliferation occurred in T-reg precursors with low Leptin receptor density, high rates of apoptosis occurred in T-reg precursors with elevated Bad, Caspase 3, and CD3 expression. Despite a differential T-reg thymic output, the size of peripheral $CD4^{+}25^{hi}Foxp3^{+}$ T-reg subset was normalized regardless the genetic background. Conversely, the T-reg suppressogenicity as tested in a model of autoimmune diabetes was directly correlated with the level of Foxp3 expression. These findings lay emphasis on a multifarious genetic control on the thymic differentiation and function of T-reg cells independently of the MHC class II-peptide presentation.

Introduction

The "professional" $CD4^{+}25^{hi}$ T regulatory cells (T-regs) represent a subset of T lymphocytes that differentiate in thymus as the "guardians" of the immune system.[1] The T-regs control the immunoreactivity to *self* and foreign antigens and at the same time maintain homeostasis of T-cell compartment in peripheral lymphoid organs.[2-5] Although the $CD4^{+}25^{hi}$ T-regs represent only 5-10% of the entire CD4 T-cell population, subtle alterations in their frequency and/or function associate with high incidence of autoimmune disorders.[6-12]

The $CD4^{+}25^{hi}$ T-reg cells express a high density of IL-2Rα chain (CD25) on the cell surface.[13] They are anergic by themselves, and do not secrete IL-2, although they require IL-2 and TCR stimulation and costimulation for development and expansion in periphery.[14-17] Several other surface receptors such as CTLA-4, OX40 (TNFRSF4), glucocorticoid induced TNF receptor super family member (GITR), TNFR2, 4-1BB, PD1, CD103 (αEβ7 integrin), Toll Like Receptors (TLR), and the recently described galectin-10, are also expressed by $CD4^{+}25^{high}$ T-reg cells.[18-22] However, the Foxp3 master regulatory gene encoding for Scurfin (SFN) is preferentially expressed in $CD4^{+}25^{hi}$ T-reg cells.[23,24] Scurfin is a fork head-winged helix transcription factor [25], and its mutated variant was associated with X-linked autoimmune disorders in *scurfy* mutant mice, and IPEX/XLAAD (Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked) in humans due to alterations in the T-reg cell compartment.[26]

Studies on the thymic development of T-cells showed that some 12 days after gestation the bone marrow-derived T cell precursors migrate at the thymic cortico-medullary junction as triple negative $CD3^{-}4^{-}8^{-}$ cells.[27,28] As they progress to the double

negative DN1 ($CD4^-8^-44^+25^-$) and DN2 ($CD4^-8^-44^+25^+$) stages, they enter the subcapsular cortex as DN3 ($CD4^-8^-44^-25^+$) cells, differentiate into DN4 ($CD4^-8^-44^-25^-$) cells, and further migrate to the cortex to proliferate and differentiate into double positive DP ($CD4^+8^+$) cells expressing a mature α/β heterodimer TCR on the surface. The majority of DP cells undergo a negative selection by apoptosis-induced cell death leading to elimination of *self*-reactive precursors. Fewer DP cells undergo positive selection generating single positive (SP) T cells ($CD4^+8^-$ or $CD4^-8^+$) with low or no specificity for *self*-antigens.[29] However, it is unclear whether T-reg precursors follow a similar pathway of thymic differentiation like the conventional T cell precursors.

Our study revealed that the $CD4^+25^{hi}Foxp3^+$ T-reg precursors followed different patterns of thymic differentiation in two mouse strains of different genetic backgrounds, BALB/c and B10D2, but sharing the same $H\text{-}2^d$ MHC class II haplotype. Differential development of $CD4^+25^{hi}Foxp3^+$ T-regs in these mouse strains led to variations in the thymic output and threshold of suppressogenicity of the mature $CD4^+25^{hi}$ $Foxp3^+$ T-reg pool. A dual mechanism responsible for such variations relied on the rates of proliferation and apoptosis in T-reg precursors, whilst the suppressogenic thresholds of mature $CD4^+25^{hi}$ $Foxp3^+$ T-reg cells in periphery was much related to the level of Foxp3 expression.

Materials and methods

Mice

One-month-old, female BALB/c and B10.D2 (H-2^d) mice were purchased from Jackson Laboratories. The TCR-HA and RAG^{-2}-RIP-HA transgenic mice used in adoptive cell co-transfer experiments are currently maintained in our facilities. Mice were housed at USUHS in a pathogen-free facility according to federal and local regulations.

Cell Purification

The $CD4^+$ thymocytes and splenocytes from pools of mice (n = 6) or individual mice were negatively sorted on mouse CD4-enrichment columns (R & D Systems, Minneapolis, MN) according to manufacturer's protocol. A second passage of $CD4^+$ cells on mouse CD8-enrichment columns (R & D Systems) yielded $CD4^-8^-$ double negative cells. The purity and viability was more than 90% as determined by FACS and trypan blue exclusion method, respectively. The $CD4^+25^+$ thymocytes and splenocytes were isolated on MS MACS paramagnetic columns (Miltenyi Biotech Inc., Auburn, CA). Briefly, the negatively-sorted $CD4^+8^-$ single positive or $CD4^-8^-$ double negative cells were incubated with anti-CD25-PE Ab-dye conjugate for 10 min, washed, and further incubated anti-PE Ab-microbeads for 15 min. The cells were sorted on magnetic columns (Miltenyi Biotec) into a $CD25^-$ fraction (flow through) and used in assays as T-cell responders, and into a $CD25^+$ fraction as T-reg cells (bound fraction). In some experiments, the $CD4^-8^-$ double negative thymocytes were stained with anti-CD44 and CD25 Ab-dye conjugates and sorted into DN1 ($CD4^-8^-44^+25^-$), DN2 ($CD4^-8^-44^+25^+$),

DN3 ($CD4^-8^-44^-25^+$) and DN4 ($CD4^-8^-44^-25^-$) subpopulations using a FACSAria instrument and FACSDiva analysis software (Becton Dickinson).

RT-PCR and Quantitative Real-time PCR

Total RNA and cDNA was prepared using NucleoSpin RNA II kit (BD Biosciences Clontech, Palo Alto, CA) and Qiagen One Step RT-PCR kit (Qiagen Inc., Valencia, CA), respectively, as previously described.[30] The primers for murine Foxp3 were: (forward 5'CAGCTGCCTACAGTGCCCCTAG3', and reverse 5'CATTTGCCAGCAGTGGGTAG3'; [25] Fas (forward CGCTGTTTTCCCTTGCTGCAG, reverse ACAGGTTGGTGTACCCCCAT),[31] Fas-L (forward CACTGACCCCTCTAAAGAAGAA, reverse TTGAATACTGCCCCCAGGTA).[32] The specific primers for Bcl-2, Bcl-X_L, Bad, Bax, and Caspase 3 were used as previously described.[30] Four nanograms of total RNA were required to prepare first-strand cDNA using a Qiagen One Step RT-PCR kit (Qiagen, Valencia, CA) at 35 and 40 cycles of amplification following the manufacturer's protocol. 25μL of each PCR amplicon was electrophoresed in 1.5% agarose gel containing ethidium bromide, and the percent of transcripts was calculated by Scion Image software analysis (Scion Corp., Frederick, MD) based on the integration of the percent of pixels and band intensity after normalization against the corresponding β-actin band, as we previously described.[30] To measure the expression level of Foxp3 by quantitative real–time RT-PCR, total RNA was extracted from mouse lymphocytes using Total RNA Purification System (Invitrogen). 500 ng RNA was used to synthesize the first cDNA strand with the High Capacity cDNA Archive kit (Applied Biosystems). PCR

amplification mixtures contained Applied Biosystems Universal PCR MasterMix Buffer (25 μl), cDNA template (22.5 μl), Foxp3 and 18S rRNA primers (2.5 μl), respectively. Quantitative RT-PCR measurements were performed on an ABI Prism 7700 with SDS 1.9.1 Software (Applied Biosystems). The melting-curve analysis showed the specificity of the amplifications. The relative mRNA levels were estimated by the standard method using 18S rRNA as the reference gene.

Flow Cytofluorimetry

Thymocytes or splenocytes ($2x10^5$) were surface, intra-cellularly, or intra-nuclearly stained with various antibody-dye conjugates or their isotype controls (BD PharMingen, San Diego, CA), or Annexin V-PE conjugate according to the manufacturer's instructions. Between 200,000 and 500,000 cell events were acquired on a LSR II Bekton-Dikinson instrument for each preparation. For accurate measurements of mean fluorescence intensity (MFI) at the single-cell level, the slight difference in the size and signal-to-noise autofluorescence of CD4 splenocytes were compensated by WINlist analysis software 3D 5.0 (Verity Software House, Topsham, ME) during the data acquisition by FACS. Measured parameters were compared under various experimental conditions using the Sigmaplot analysis software v. 8 (Aldrich-Sigma). Overall data were presented for convenience as mean ± SD of pooled samples, or independently as representative experiments.

In Vivo Cell Cycle Division

Mice were. injected intraperitoneally (i.p) with 1 mg of carboxyfluorescein succinimidyl ester (CFSE) in saline, and four and six days later some 1 x 10^6 gated $CD4^+CD25^{hi}$ T-reg cells isolated from the thymus and spleen were harvested and data were acquired using a LSR II instrument (BD Biosciences). The pattern and number of cell cycle divisions based on the CFSE dilution factor were analyzed using the WINlist software 3D 5.0.

Caspase 3 Activity

25 μg of protein extract from $CD4^-8^-$ double negative thymocytes isolated as described, were incubated for 40 min in 96-well plates in 100 μl buffer (100 mM HEPES, 10% sucrose, 0.15% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin, pH 7.4) supplemented with 1 μM of substrate (Ac-DEVD-AMC, Peptide Intern., Louisville, KY) at 37^0C. The amount of substrate released was measured in a spectrofluorimeter (excitation at λ = 360nm and emission at λ = 4360nm) equipped with the XFluor analysis software (Tecan US, Durham, NC). Standard dilutions of the substrate were used in each assay to measure the absolute concentration of released AMC, and the measured fluorescence units were converted into rate of AMC cleavage per mg protein (corresponding to pmole cleaved DEVD-AMC x min^{-1} x mg^{-1}). Values were compensated for the signal-to-noise background (spontaneous release of AMC in the absence of cell extract)

Adoptive Cell Transfers

The RAG2 KO, RIP-HA Tg mice (BALB/c x B10D2, H-2^d background) expressing the hemagglutinin protein (HA) of influenza PR8 virus in the pancreatic β-cells under the rat insulin promoter[10] were used as hosts for splenic $CD4^+25^{hi}$ $Foxp3^+$ T-reg cells (10^5 cells) from BALB/c or B10.D2 mice, and diabetogenic, HA-specific $CD4^+25^-$ T-cells (2.5 x 10^5 cells) from BALB/c mice transgenic for a T cell receptor (TCR) that recognizes the HA110-120 immunodominant CD4 T-cell epitope of HA of PR8 influenza virus (TCR-HA Tg mice).[33] The TCR-HA Tg mouse expresses the TCR-HA transgene on 35-40% of splenic CD4 T-cells. Intraperitoneal infusion (i.p.) of HA-specific $CD4^+25^-$ T-cells from TCR-HA Tg mice (2.5 x 10^5 cells) in RAG2 KO, RIP-HA Tg mice induces a fulminate diabetes within 10-14 days. T-regs and diabetogenic T-cells were isolated on CD4 columns followed by CD25 Ab-magnetic beads (Miltenyi Biotec, NJ) as described above. The T-reg cells were infused i.p. 7 days prior infusion of diabetogenic TCR-HA T-cells.

Diabetes Monitoring

Glycemia in the RAG2 KO, RIP-HA Tg recipients of T-regs and diabetogenic T-cells was monitored by-weekly starting after the transfer of diabetogenic T-cells and using Accu-Check Advantage glucose strips (Boehringer Manheim, Indianopolis, IN). Mice were considered diabetic when glycemia went for two consecutive measurements over 200 mg/dl. One week after the transfer of diabetogenic T-cells, 2 pancreases were collected from each group of mice, and analyzed histochemically for the presence of lymphocyte infiltration in β-islets using the conventional hematoxilin-eosin (HE) staining

of paraffin-embedded pancreatic cross sections. Survival in the RAG^{-2} RIP-HA Tg recipient mice was monitored on a daily basis.

Statistic Analysis

Significance of survival of Rag^{-2}RIP-HA mice co-infused with T-reg cells and diabetogenic T cells among control groups was determine by Kaplan-Meier test. The significance of individual differences in MFI and percentage in mice analyzed by FACS was determined by Student's *t*-test.

Results

$CD4^-8^-$ stage of thymic differentiation is the earliest time-point of Foxp3 expression

Foxp3 transcripts were previously detected in BALB/c mice by RT-PCR in $CD4^+8^+$ double positive (DP) and $CD4^+8^-$ single positive (SP4) thymocytes, but not in $CD4^-8^-$ double negative (DN) thymocytes.[25] Herein, we analyzed the kinetics of Foxp3 mRNA and Foxp3 protein expression in the T-reg thymic precursors from BALB/c and B10D2 mice by RT-PCR and real-time RT-PCR. The B10.D2 recombinant inbreed mouse has a C57BL/6 background and shares the same MHC class II haplotype ($H\text{-}2^d$) with the BALB/c mouse. Consistent with previous findings, Foxp3 mRNA was absent in the BALB/c DN thymocytes when analyzed by RT-PCR, but present in the B10.D2 DN thymocytes (Figure 1A, upper panel). In contrast, real-time RT-PCR revealed the presence of Foxp3 transcripts early in the B10.D2 DN thymocytes (Figures 1A and 1B),

and at lesser extent in the BALB/c DN thymocytes (Figure 1B). Consistent with previous reports, the majority of Foxp3-expressing thymocytes were found in the SP4 population.

These results showed that Foxp3 message is expressed early in the $CD4^-8^-$ stage of thymic differentiation in two different mouse strains.

Foxp3 is differentially expressed in the T-reg precursors from BALB/c and B10.D2 mice

FACS-sorted DN1-4 thymocytes (Figure 1C) were analyzed for Foxp3 expression by quantitative real-time RT-PCR and FACS intracellular staining. The Foxp3 transcript and protein was present only in the DN3 and DN4 thymocytes regardless the genetic background (Figure 1D). Interestingly, more than 90% of DN3 thymocytes in both mouse strains expressed Foxp3 protein as compared with 8-9% of DN4 thymocytes. A quite significant difference between the BALB/c and B10.D2 mice was observed during the early thymic differentiation, as the number of SP4 thymocytes expressing Foxp3 was 3-times higher in the B10.D2 mice (Figure 1E).

These results showed an early Foxp3 expression in the DN3 and DN4 stages of thymic differentiation in both mouse strains, although at an overall higher level in the B10.D2 mice. Higher level of Foxp3 expression in B10.D2 mice than in BALB/c mice indicated that this quantitative difference is controlled by genetic elements independently of the MHC class II genes, since both mouse strains share the same $H\text{-}2^d$ haplotype.

T-reg precursors proliferate at different rates in the thymus of BALB/c and B10.D2 mice

To find out whether the rates of $CD4^{+}25^{hi}$ T-reg thymic proliferation could affect the thymic output, we compared the number of cell cycle divisions of T-reg thymic precursors in these mice using the CFSE dilution factor *in vivo*. Our pilot experiments showed that intravenous injection of CFSE in mice provides accurate measurements of the *in vivo* cell cycle division in many cell types including the thymocytes, and it lacks toxicity at a quite high dose of administration (ex. 1 mg per mouse)(data not shown). The BALB/c and B10.D2 thymocytes showed equal uptake of CFSE *in vivo* and *in vitro* some 30 min post injection (Figure 2A, lower panel). The $CD25^{hi}$ expression has been chosen in these experiments instead of Foxp3 expression to identify the T-reg precursors, since the later approach requires intra-nuclear staining in a 2-day procedure, and this could overwrite the dilution factor of CFSE fluorescence. As previously reported, and by us in pilot experiments, more than 90% of $CD4^{+}CD25^{hi}$ cells showed Foxp3 expression.

The BALB/c' DP (25^{hi}) and SP4 (25^{hi}) thymocytes exhibited a lower rate of proliferation in thymus (4-5 cycles of division on day 4, and 5-6 cycles of division on day 6 post-CFSE-labeling) as compared with their B10.D2' counterparts (5-6 cycles on day 4, and 7-8 cycles on day 6 post-CFSE-labeling) (Figure 2A, upper panels).

Both the mature $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg cells and conventional $CD4^{+}25^{-}$ T-cells express leptin receptor (Obese Receptor, ObR). The ObR provides a negative signal for proliferation in $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg cells.[21] Accordingly, the T-reg ObR^{low} cells proliferate at faster rates than the T-reg ObR^{hi} cells. Our FACS single-cell level analysis revealed that the ObR partitioning on BALB/c and B10.D2 $CD4^{+}25^{hi}$ T-reg progenitors

followed similar patterns. In both strains, the DN thymocytes showed a lower ObR expression level than the DP CD25^{hi} thymocytes, and the terminally differentiated SP4 25^{hi} T-reg thymocytes showed the presence of both ObR^{low} and ObR^{hi} cell populations (Figure 2B). However, the number of SP4 25^{hi} ObR^{low} T-reg precursors in BALB/c mice was 2-2$^{1/2}$-times lower than in the B10.D2 mice. Our data showing a lower rate of thymic proliferation of BALB/c' CD4^{+}25^{hi} T-reg precursors *in vivo* than their B10.D2 counterparts (Figure 2A), were consistent with previous findings indicating a higher number of slow-dividing (CD4^{+}25^{hi} ObR^{hi}) T-reg precursors in BALB/c mice (89 ± 5.1%) as compared with B10.D2 mice (74 ± 4.3%) (Figure 2B). A differential rate of proliferation of BALB/c *vs*. B10.D2 T-reg precursors was confirmed also by the 1$^{1/2}$-2-times higher mRNA expression of Cdk4 in B10.D2 T-reg precursors, a cyclin protein actively involved in the transition of cells from G_0 to S_1 phase. There was no difference between the BALB/c and B10.D2 T-reg precursors in the mRNA expression level of p27^{kip}, a major cyclin inhibitor (Figure 2C).

Together, these results strongly suggest that the differential rates of T-reg thymic proliferation in BALB/c and B10.D2 mice are likely to affect at least at some extend the thymic output of CD4^{+}25^{hi}Foxp3^{+} T-reg cells. Lower rates of thymic proliferation of T-reg precursors in BALB/c mice occurred in the context of a higher number of slow-dividing (ObR^{hi}) T-reg precursors.

T-reg precursors die at different rates in the thymus of BALB/c and B10.D2 mice

We next addressed the thymic negative selection as a possible mechanism leading to the differential CD4^{+}25^{hi}Foxp3^{+} thymic output in the BALB/c and B10.D2 mice. The

number of dying Foxp3$^+$ T-reg precursors was different in all stages of thymic differentiation in BALB/c and B10.D2 mice (Figure 3A). The BALB/c T-reg precursors showed a 4-fold higher Annexin V binding in the DN stage, and more than 2-fold increase in the DP and SP4 stages. A significantly high number of apoptotic Foxp3$^+$ thymocytes (~50%) were detected early in the DN3 stage in BALB/c mice as compared with only 4.5% in the B10.D2 mice (Figure 3B). The differential rates of apoptosis in T-reg precursors were well correlated with the basal level of Bad and Caspase 3 expression, i.e., 3-times higher basal level of Bad and Caspase 3 mRNA and protein expression, and 2-times higher Caspase 3 activity in the CD4$^+$CD25^{hi} T-reg precursors of BALB/c mice as compared with B10.D2 mice (Figure 3C). There were no significant differences in the mRNA and protein expression levels of Bcl2, Bcl-X_L, Bax, Fas, and FasL in the CD4$^+$CD25^{hi} thymocytes between these two mouse strains.

In conclusion, a differential rate of cell death in Foxp3$^+$ T-reg precursors may also explain the variations in the thymic output of CD4$^+$25$^+$Foxp3$^+$ T-reg cells between the BALB/c and B10.D2 mice. Of note, the DN stage was not only the earliest time-point of Foxp3 expression (Figure 1), but most likely the earliest check-point for negative selection of T-reg precursors. Overall, the BALB/c T-reg precursors showed a greater cell death than their B10.D2 counterparts through all stages of thymic differentiation, which may well explain a lower CD4$^+$25$^+$Foxp3$^+$ T-reg thymic output in BALB/c mice.

The CD3 strength of stimulation controls the sensitivity of BALB/c and B10.D2 T-reg precursors to apoptosis

It has been postulated that $CD4^{+}25^{hi}$ T-reg cells are positively selected in thymus upon TCR stimulation with high-affinity *self*-peptides presented by thymic epithelial cells (TEC). Conversely, for an insufficient TCR stimulation such as below the threshold of high-affinity recognition, the T-reg precursors undergo negative selection.[34,35] In addition; the cell density of CD3/TCR complex can also dictate the strength of stimulation. Higher CD3/TCR cell density and thereby, higher number of CD3/TCR molecules interacting with the MHC II-peptide complexes can provide stronger intracellular signals for activation as well as for apoptosis. To this, CD28 costimulation provides an enhanced and prolonged CD3/TCR signaling.

We first compared the level of CD3 and CD28 expression on T-reg precursors in both mouse strains by FACS at the single-cell level. The CD3, but not the CD28 expression was 2-times higher on DN thymocytes, and more than 3-times higher on DP $CD25^{hi}$ thymocytes from BALB/c mice than B10.D2 mice (Figure 4A). In contrast, the SP4 $CD25^{hi}$ thymocytes showed a similar expression of CD3 and CD28 receptors in both mouse strains. Similar CD3 and CD28 cell density was detected in SP4 $CD25^{hi}$ thymocytes when Foxp3 protein was used as a T-reg tag in FACS intracellular staining (data not shown).

We next compared the sensitivity to apoptosis of $CD25^{hi}$ T-reg precursors from BALB/c and B10.D2 mice at different strengths of stimulation by using various doses of CD3 and CD28 Abs. The CD3 antibodies were used as a surrogate of TCR stimulation. Stimulation of T-reg precursors with different doses of CD3/CD28 antibodies reduced the

number of Annexin V^+ T-reg precursors in a dose-dependent manner in both mouse strains (Figure 4B), whereas the CD28 antibodies alone did not (data not shown). However, the BALB/c T-reg precursors showed an overall increased sensitivity to CD3-induced apoptosis as compared to their B10.D2 counterparts. Although no significant difference in the CD3 density per cell was observed between the SP4 T-reg precursors from BALB/c and B10.D2 mice, the percent of apoptotic cells was higher in BALB/c T-reg precursors at any of the CD3 Ab doses used in experiment. This may accounted for a higher Bad and Caspase 3 expression in BALB/c T-reg precursors as compared to B10.D2 mice (Figure 3C).

These results indicated that the $CD25^{hi}$ T-reg precursors from BALB/c mice showed constantly a 2 to 3-fold increase in the CD3/TCR propensity in direct relation to a higher sensitivity to CD3-induced apoptosis as compared with their B10.D2 counterparts. In contrast, the strength of CD28 stimulation did not affect the threshold of CD3-induced apoptosis in BALB/c and B10.D2 T-reg precursors.

Differential T-reg thymic outputs are homeostatically normalized in periphery

Since the number of terminally differentiated $CD4^+8^-25^{hi}$ $Foxp3^+$ T-reg thymocytes was 3-times lower in BALB/c than in B10.D2 mice (1.54 ± 0.67% *vs*. 4.15 ±. 0.91%), we next questioned whether variations in the thymic output of T-reg cells might have an impact on the size of mature $CD4^+25^{hi}$ $Foxp3^+$ T-reg pool in peripheral lymphoid organs. The frequency of $CD4^+25^{hi}$ $Foxp3^+$ T-regs in the spleen of BALB/c and B10.D2 mice was in a close range (6.8 ± 1.4% and 7.5 ± 1.2% respectively), implying that, regardless the thymic output, a compensatory homeostatic mechanism operates efficiently

to normalize the size of peripheral T-reg compartment. Indeed, the analysis of cell cycle divisions of splenic $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-regs from CSFE-labeled mice showed a quite different proliferation rate *in vivo* between the two mouse strains. While the mature, splenic $CD4^{+}25^{hi}$ T-reg cells underwent 4-5 cycles of homeostatic division within a 4-day interval, and 7 cycles at the end of day 6 post CFSE injection, a significant number of splenic $CD4^{+}25^{hi}$ T-reg cells from B10.D2 mice (32-35%) were slow-dividing cells (2-3 cycles of division within 6 days) (Figure 5A). This may account for an overall slower homeostatic proliferation of splenic $CD4^{+}25^{hi}$ T-reg cells in B10.D2 mice leading to a similar number of mature T-regs as in the BALB/c mice. Noteworthy, the mice investigated in this study were housed in a pathogen-free barrier, which rules out the possibility of opportunistic infections that may affect T-reg homeostasis.

The ObR propensity on T-reg cells controls their thresholds of proliferation[21], and we found a difference in the ObR partitioning on T-reg thymic precursors from BALB/c and B10.D2 mice. Although the serum leptin concentration did not differ significantly (BALB/c, 4.5 ± 0.4 μg/ml and B10D2, 4.8 ± 0.6 μg/ml, n = 7 mice per group), the B10.D2 mice showed constantly a major, slow-dividing $CD4^{+}25^{hi}$ ObR^{hi} T-reg cell population (Figure 5B).

Together, these results suggest that the $CD4^{+}25^{hi}$ T-reg thymic output does not affect the size of peripheral, mature T-reg compartment, whereas the ObR propensity on mature T-reg cells may play an important role on their homeostatic proliferation.

The pattern of T-reg development in thymus echoes their suppressogenic threshold

As the differential thymic development of $CD4^{+}25^{hi}$ T-reg cells in these two different genetic backgrounds did not affect the size of peripheral T-reg compartment, we next questioned whether the suppressogenic function of mature, peripheral $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg cells might be affected.

In an *in vitro* approach, the suppressogenic capacity of splenic $CD4^{+}25^{hi}$ T-reg cells on the function of syngeneic $CD4^{+}25^{-}$ T responder cells was tested by measuring (1) the mRNA level of Th1 and Th2 transcription factors, and (2) the ability of T responder cells to secrete cytokine upon stimulation for 30 h with CD3 and CD28 antibodies. At the 1:1 ratio of T-regs to T responder cells, the B10D2 T-regs inhibited almost 2-times better the IL-2 and IFN-γ secretion by T responder cells than the BALB/c T-regs (Figure 6A, upper panel). The mRNA levels encoding for Th1 and Th2 transcription factors T-bet, STAT4, NFATc, STAT6, GATA3, and cMAF in T responder cells were also lower upon suppression by B10.D2 T-regs as compared to those suppressed by BALB/c T-regs (Figure 6A, lower panel). Our *in vitro* system for assessment of T-reg suppressogenicity strongly suggested that the B10.D2 T-regs are more potent than their BALB/c counterparts.

To assess the suppressogenicity of splenic $CD4^{+}25^{hi}$ T-reg cells from BALB/c and B10.D2 mice *in vivo*, we took advantage of an experimental autoimmune model for type 1 diabetes (T1D). In this system, RAG2 KO, RIP-HA Tg mice ($H\text{-}2^{d}$) expressing the influenza HA viral antigen in the pancreatic β-islets under the rat insulin promoter develop a rampant hyperglycemia 10-12 days after infusion with diabetogenic (HA-specific) CD4 T-cells from mice bearing a TCR-HA transgene (TCR-HA Tg T-cells/H-

2^d), and they die within the next two weeks of hyperglycemia.[10] The suppressogenicity of splenic $CD4^+25^{hi}$ $Foxp3^+$ T-reg cells from BALB/c and B10.D2 mice was tested in relation to their ability to delay or prevent the onset of autoimmune diabetes in RAG2 KO, RIP-HA Tg recipients, and to alter their survival index upon co-transfer with diabetogenic T-cells. At the 1 to 2.5 ratio of T-reg to TCR-HA T diabetogenic cells, the T-reg cells from both B10.D2 and BALB/c mice were unable to delay significantly the onset of hyperglycemia in RAG2 KO, RIP-HA Tg mice (Figure 6B**,** upper panel). However, only the T-reg cells from B10.D2 mice prolonged the survival index of RAG2 KO, RIP-HA hyperglycemic recipients (Figure 6B, lower panel), i.e., 80 % of hyperglycemic recipients survived for 1 month after the onset of hyperglycemia. Prolonged survival occurred in the context of a protective peri-islet pancreatic infiltration with lymphocytes (Figure 6C). In contrast, only 50% of the hyperglycemic recipient mice co-infused with T-regs from BALB/c mice and diabetogenic T-cells survived two more weeks after the onset of hyperglycemia, and displayed a destructive (intra-islet) β-islet infiltration. The number of heavily infiltrated β-islets in diabetic mice receiving T-regs from B10.D2 mice (10 ±2 of 20 scored islets per pancreas from 3 mice) was lower than in those receiving T-regs from BALB/c mice (16 ± 3 of 20 scored islets per pancreas from 3 mice). These experiments clearly indicated that the non-antigen-specific B10.D2 T-reg cells provided a better protection against pancreatic infiltration with lymphocytes than their BALB/c counterparts.

A stronger suppressogenic function of mature, splenic B10.D2 T-reg cells occurred in the context of a higher content of Foxp3 protein than in BALB/c T-regs as determined by FACS intracellular staining at the single-cell level (MFI = 307 ± 14 *vs*.

135 ± 12). This was consistent with the real-time RT-PCR data showing that the level of Foxp3 mRNA expression was 1.5-2-times higher in the splenic B10.D2 T-reg cells. These results indicated that a differential thymic development of $CD4^+25^{hi}$ T-reg cells as detected in two different genetic backgrounds, was associated with a differential suppressogenic capacity of mature, peripheral $CD4^+25^{hi}$ $Foxp3^+$ T-reg cells.

Discussion

The cellular and molecular events leading to thymic differentiation of T-reg cells are still elusive. Foxp3 has lately incited much interest as the master regulatory gene of thymic development and function of $CD4^+25^{hi}$ T-reg cells. Foxp3 transcripts were previously detected in $CD4^+8^+$ double positive and $CD4^+8^-$ single positive thymocytes.[25] Using sensitive, quantitative real-time RT-PCR and FACS intranuclear staining assays, we detected Foxp3 mRNA and protein early during the DN3 and DN4 stages of thymic differentiation in two different mouse strains, BALB/c and B10.D2 mice, which share the same $H\text{-}2^d$ MHC class II haplotype. Foxp3 expression followed different kinetics through all stages of thymic differentiation.

Along with a differential Foxp3 expression in T-reg precursors, the size of $Foxp3^+$ thymic pool varied at each stage of differentiation in BALB/c and B10.D2 mice. Differential $CD4^+25^+$ thymic output between B6, DBA/2 and SJL inbred mice bearing different MHC class II haplotypes has been also described.[36] We found a dual mechanism leading to a differential $CD4^+25^{hi}$ $Foxp3^+$ T-reg development and thymic output in BALB/c *vs*. B10.D2 mice. First, the B10.D2 T-reg precursors proliferated more vigorous in the thymus than their BALB/c counterparts. Higher rates of proliferation were

paralleled by an increased basal level of Cdk4, and a larger number of fast-dividing (ObR^{low}) $Foxp3^+$ thymocytes. The ObR cell propensity has also delineated a new phenotypic marker for mature $CD4^+25^{hi}$ T-reg cells, since the overall ObR density at the single-cell level was 5 to 6-times higher in splenic T-reg cells than in conventional splenic $CD4^+25^-$ T-cells in both mouse strains (Figure S1, online). Since the ObR provides negative signaling for cell proliferation, this may explain why the T-reg cells represent only a fraction (5-10%) of the entire T-cell compartment.

A second arm of this dual mechanism leading to a differential $CD4^+25^{hi}$ $Foxp3^+$ T-reg thymic output in BALB/c *vs*. B10.D2 mice refers to the extent of thymic selection of T-reg cells. Higher rates of T-reg apoptosis in BALB/c mice than in B10D2 mice were paralleled by an increased basal level of Bad and Caspase 3 expression. The role of apoptosis in thymic negative selection of conventional T-cells is still a matter of debate, and little is known about the role of apoptosis in thymic selection of T-reg cells. The present study revealed a role of Bad and Caspase 3 in T-reg apoptosis. It remains to be investigated whether the expression of pro-apoptotic proteins in T-reg precursors such as Bad and Caspase 3 is genetically programmed, or post-translational regulated. It has been also shown in transgenic mice expressing an agonistic *self*-peptide in thymus or a peptide-specific TCR, that the antigen-specific T-reg precursors require a stronger CD3/TCR stimulation than conventional T-cell precursors for positive selection in thymus.[34,35] We found that a higher CD3, but not CD28 propensity in T-reg precursors was paralleled by a lower threshold of CD3-induced apoptosis in both mouse strains and at all stages of thymic differentiation including the DN3/4 stages. The DN3/4 transition appeared to be not only the earliest time-point of Foxp3 expression in T-reg precursors,

but also their earliest check-point for negative selection. Indeed, the thymic cortical area where most of the DN thymocytes reside, displays a high extend of cell death.[37] It is not surprising that the DN thymocytes can be signaled toward CD3-induced apoptosis in the absence of a CD4 receptor, since $p56^{lck}$ phosphorylation and segregation of pre-TCRα in the plasma membrane lipid rafts occurs in DN thymocytes as well.[38]

A differential Foxp3 expression and thymic development of T-reg cells in BALB/c and B10.D2 mice did not affect the size of splenic $CD4^{+}25^{hi}$ T-reg pool. An efficient homeostatic mechanism operated efficiently to normalize the size of peripheral $CD4^{+}25^{hi}$ T-reg pool. In contrast to the rate of T-reg thymic proliferation, the rate of splenic proliferation of $CD4^{+}25^{hi}$ T-regs in BALB/c mice was more accelerated than in B10.D2 mice, which may well account for a larger number of fast-dividing (ObR^{low}) $CD4^{+}25^{hi}$ T-reg cells in BALB/c mice (58% *vs*. 41%). However, in contrast to the size of peripheral T-reg pool, the T-reg suppressogenic capacity was different in these two strains. As tested in a model of autoimmune diabetes, the splenic $CD4^{+}25^{hi}$ T-regs from B10.D2 mice were more suppressogenic and at the same time expressed more Foxp3 than their BALB/c counterparts. Several reports suggested that higher Foxp3 expression correlates with higher T-reg suppressogenicity, but little is known about the signaling pathways that control Foxp3 expression and how Foxp3 modulates the suppressogenicity thresholds in T-reg cells. An epigenetic control of Foxp3 expression was suggested based on the polymorphism in Foxp3 binding sites for transcription factors like ATF/CREB, C/EBP-γ, Elk-1, Ets-1, GATA-4, NFATc, NF-kB, SMAD-4, STAT-1, TGF-4, and TTF1.[39] However, epigenetic alterations may affect not only the Foxp3 *locus*, but also *loc*i encoding modulators of cell cycle division and apoptosis in T-reg precursors. More

than 700 genes have been recently identified to be up- and down-regulated in developing and mature $CD4^{+}25^{hi}$ T-reg cells from C57BL/6 mice.[40] Several polymorphic genes with unique expression in different genetic backgrounds were found to affect the thymic differentiation of T-reg cells.[36] A genetic polymorphism in the Foxp3 binding sites for several transcription factors has been also described.[39] Inasmuch as DNA methylation events were shown to assist chromatin-remodeling and the circadian clock-regulated gene expression during differentiation of T-cells into Th1 and Th2 cell lineages,[41,42] similar events may play a role in T-reg thymic differentiation. The 1st and 2nd amplicon of Foxp3 promoter located upstream of exon 1 is the most susceptible region for DNA methylation.[39] Our analysis on bisulfite-mediated DNA methylation in $CD4^{+}25^{hi}$ T-reg precursors ruled out the possibility that variations in *Foxp3* expression during T-reg thymic differentiation between the BALB/c and B10.D2 mice could be explained by a differential DNA methylation in the *Foxp3* gene promoter of T-reg precursors. We found an equal number of fifteen CpG sites of methylation in the 1st and 2nd amplicon of Foxp3 promoter in the BALB/c and B10.D2 thymocytes (Figure S2, online). Also, this region showed 100% homology between the two mouse strains. In contrast, DNA methylation in the 1st *Foxp3* intron was recently described to be inversely correlated with the binding of CREB/ATF complex transcription factor and Foxp3 expression.[43] It remains to be determined whether various extends of methylation in the *Foxp3* non-coding regions may lead to a differential Foxp3 expression.

All together, our findings revealed a fine tuning of key regulators of thymic proliferation, differentiation, and selection in T-reg precursors such as CD3, ObR, Cdk4, Bad, and Caspase 3, which argues for a genetic and/or epigenetic control of T-reg

development independently of the MHC class II-peptide presentation. Since T-reg cells play an important role in the immune homeostasis and in several pathological conditions, the status of T-reg compartment referred as to its size and suppressogenic function can be responsible at least in part for variations in the susceptibility to infections, cancer, and autoimmune disorders. It remains to be investigated whether a differential development and function of T-reg compartment as found in the murine systems, can be also identified in individuals from various ethnic groups. Recently, correlates have been reported between the genetic polymorphism at (GT)n, (TC)n1, DXS573, and DXS120B *loci* in Foxp3 gene, and variations in susceptibility to autoimmune thyroiditis in Caucasian and Japanese groups.[44]

Supplemental Data

Figure S1. ObR Partitioning on Conventional T-cell Thymic Precursors and Mature $CD4^+25^-$ T-cells

Partitioning of leptin receptor (ObR) on $CD25^-$ thymocytes and $CD4^+25^-$ splenocytes from BALB/c and B10.D2 mice was determined by single-cell FACS analysis. Cells were stained with CD4-PE-Cy7, CD25-APC, and ObR-FITC Ab-conjugates, and the ObR mean fluorescence intensity (MFI) was measured in the $CD25^-$ gated DP or SP4 thymocytes, and in the $CD4^+25^-$ gated splenocytes. Shown are the MFI ± SD values (bold), and percent of each cell population from individual mice (n = 4 mice per group) expressing low and high ObR density on the surface. For the accuracy in comparison of

T-reg and conventional T-cells in terms of ObR partitioning,, the same mice described in figures 2 and 5 were also analyzed in this experiment.

Figure S2. Frequency of CpG Motifs in the Amplicons 1 and 2 of Foxp3 Gene Promoter from BALB/c and B10.D2 Thymocytes

Individual CpG motifs are shown as bold/underlined base pairs. Bisulfite sequencing and alignment of BALB/c and B10D2 genomic sequences corresponding to the overlapping amplicons 1 and 2 in the foxp3 gene promoter was carried out as follows: genomic DNA was isolated from purified T cells of 1 month-old mice BALB/c and B10.D2 mice using the DNeasy tissue kit (Qiagen, Valencia, California, United States) and following the supplier's recommendations. Sodium bisulphite treatment of genomic DNA was performed as previously described,[39] with minor modifications aimed at deamination of unmethylated cytosines to uracil, whereas methylated cytosines remained unchanged. In a subsequent PCR amplification, uracils were replicated as thymidines. Thus, detection of a ''C'' in sequencing reactions reflects methylation of the genomic DNA at that site. Detection of a ''T'' at the same site reflects instead the absence of a methyl modification of the genomic cytosine. PCRs were performed in a final volume of 25 μl containing 13 μl PCR buffer, 1-U Taq DNA polymerase (Qiagen), 200 lM dNTPs, 12.5 pmol each of forward and reverse primers, and 7 ng of bisulphate treated genomic DNA. The amplification conditions were 95^{o} C for 15 min and 40 cycles of 95^{o} C for 1 min, 55^{o} C for 45 sec, and 72^{o} C for 1 min, and a final extension step of 10 min at 72^{o} C. PCR products were purified using Qiagen MinElute gel extraction kit and sequenced in both directions applying the PCR primers and the ABI Big Dye Terminator v1.1 cycle

sequencing chemistry (Applied Biosystems, Foster City, California, United States), followed by capillary electrophoresis on an ABI 3100 genetic analyzer. For each sample, both PCR amplification and sequencing was repeated once. The following primers specific for the murine Foxp3 gene promoter (5' to 3' direction) were used for both PCR amplification of bisulphite converted genomic DNA and sequence reactions: Amp 1 (f: 5'AGGAAGAGAAGGGGGTAGATA; r: 5'AAACTAACATTCCAAAACCAAC), Amp 2 (f: 5'ATTTGAATTGGATATGGTTTGT; r: 5'AACCTTAAACCCCTCTAACATC).[39]

Acknowledgements

This work was supported by grants from the National Institutes of Health, DK61927 & DK61326 to T-D.B. and DK077521 to S.C, and JDRF-12002-1151 to SC and T-D.B. We thank Mrs Karen Wolcott and Kateryna Lund for technical assistance in FACS sorting of T-reg precursors, and to Dr. H. Zaghouani for helpful discussions. The authors declare no competing financial conflict of interest.

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Figure Legends

Figure 1. Differential expression of Foxp3 mRNA in T-reg thymic precursors from BALB/c (H-2^d) and B10.D2 (H-2^d) mice

(A) $CD4^-8^-$ (DN), $CD4^+8^+$ (DP), and $CD4^+8^-$ (SP4) thymocytes analyzed by RT-PCR for Foxp3 mRNA expression. Shown are the Foxp3 transcripts at 40 cycles of PCR amplification for a representative BALB/c and B10.D2 mouse. Similar results were obtained in other five mice. The 35 cycles PCR amplification could not detect Foxp3 transcripts in the DN thymocytes.

(B) Quantitative measurements of Foxp3 transcript by real-time RT-PCR. The relative expression level of Foxp3 mRNA (Y-axis) was calculated in relation to the lowest base level of Foxp3 mRNA as found in the BALB/c DN thymocytes. Shown are the mean values ± SD from six individual mice in each group.

(C) FACS sorting of DN1-4 thymic subpopulations. Gated DN thymocytes were stained with CD4 and CD8 PerCP-Ab conjugates, and sorted based on CD44-FITC and CD25-APC Ab staining.

(D) RNA extracted from FACS-sorted DN1-4 thymic populations as described in panel C, was analyzed by real-time PCR for the Foxp3 mRNA expression. Shown are the mean values ± SD for three individual BALB/c and B10.D2 mice. Overlapped values (red color) indicate the percent ±SD of each DN population expressing Foxp3 protein measured by FACS intranuclear staining.

(E) Thymocytes from the mice analyzed in panel (d) were cell surface stained with CD4-PE-Cy7, CD8-PerCP, and intranuclear with Foxp3-PE Ab-conjugates. Overlapped

forward scatters in each quadrant indicate the percent ±SD of total DN, DP, SP4 and SP8 thymocytes (blue color) *vs* Foxp3-expressing thymocytes (red color). The signal-to-noise background fluorescence of a rat IgG isotype PE conjugate control for Foxp3 was subtracted. Some 850,000 total cell events were acquired by a LSR II instrument and analyzed by WINList software.

Figure 2. Differential Rates of Proliferation of T-reg Thymic Precursors from BALB/c and B10.D2 mice

The mice (n = 5) were injected i.p. with CFSE, and four and six days later the thymocytes were over stained with CD4-PE-Cy7 and CD25-APC Ab-conjugates. The number of cell cycle divisions in FACS-gated $CD4^{+}25^{hi}$ thymocytes was established based on the dilution factor for CFSE fluorescence.

(A) Cell cycle divisions of FACS-gated $CD4^{+}25^{hi}$ thymocytes in a representative BALB/c and B10.D2 mouse. Empty histograms represent day 6, and dark histograms represent day 4 post CFSE injection. Lower panel shows the CFSE fluorescence after five serial, twofold dilutions *in vitro* (empty plots) as compared with the uptake of CFSE by thymocytes *in vivo* some 30 min after CFSE i.p. injection (dark plot).

(B) Partitioning of ObR on thymic populations from BALB/c and B10.D2 mice as determined by FACS using ObR-FITC Ab-conjugate (BD PharMingen). Shown are the MFI ± SD values (bold), and percent ± SD of the DN, DP $CD25^{hi}$, and SP4 $CD25^{hi}$ thymocytes from individual mice (n = 4 per group).

(C) Quantitative measurements of Cdk4 and $p27^{kip}$ mRNA expression in FACS-sorted SP4 $CD25^{hi}$ thymocytes from individual BALB/c and B10.D2 mice (n = 4 per group) as

determined by real-time PCR (specific primers, Applied Biosystems, Foster City, CA). The relative mRNA expression (Y-axis) refers to the mRNA fold increase as compared with the lowest level observed in BALB/c mice, arbitrarily considered 1 fold increase).

Figure 3. Differential Rates of Cell Death of T-reg Thymic Precursors from BALB/c and B10.D2 mice

(A) Thymocytes from BALB/c and B10.D2 mice (n = 5) were stained with CD4-FITC, CD8-APC, CD25-PerCP-Cy5.5 Ab-conjugates, and Annexin V-PE conjugate. Shown are the percent ± SD values of FACS-gated DN, DP $CD25^{hi}$, and SP4 $CD25^{hi}$ thymocytes binding Annexin V. (B) Thymocytes from the same mice as in panel A (n = 5) were stained with CD4-PerCP, CD8-PerCP, CD44-FITC, CD25-APC Ab-conjugates and Annexin V-PE conjugate. Shown are the percent ± SD of FACS-gated DN3 and DN4 thymocytes binding the Annexin V. (**c**) The mRNA and protein expression levels of anti-apoptotic and pro-apoptotic proteins in $CD25^{+}$ thymocytes from a pool of BALB/c or B10.D2 mice (n = 5) were sorted on MACS immunobeads and analyzed for Bcl-2, Bcl-X_L, Bad, Bax, Caspase 3, Fas, and FasL by RT-PCR, and by FACS intra-cellular staining. The enzymatic activity of Caspase 3 (pmole cleaved DEVD-AMC x min^{-1} x mg^{-1}) was determined by ELISA as described. The RT-PCR amplicon values at 40 cycles of amplification represent the relative mRNA expression level. The protein expression levels refer to the mean fluorescence intensity (MFI) determined by FACS intracellular staining upon subtracting the signal-to-noise background of the corresponding isotype control antibody-dye conjugates. Bolded/underlined values indicate the differential expression and activity of Bad and Caspase 3.

Figure 4. **Differential Sensitivity of BALB/c and B10.D2 T-reg Thymic Precursors to CD3-induced Apoptosis**

(A) Thymocytes from BALB/c and B10.D2 mice (n = 5) were stained with CD4-FITC, CD8-PerCP, CD25-APC, and CD3ε-PE or CD28-PE Ab-conjugates, and analyzed for the CD3 and CD28 cell density by FACS. Shown are the MFI ± SD values of CD3 and CD28 receptors on DN, DP $CD25^{hi}$ and SP4 $CD25^{hi}$ T-reg precursors.

(B) Thymocytes isolated from a pool of BALB/c and B10.D2 mice (n = 4) were stimulated for 24 h with various doses of soluble CD3ε and CD28 Abs, then washed and stained with CD4-FITC, CD8-PerCP, and CD25-APC Ab-conjugates and Annexin V-PE conjugate, and analyzed by FACS. Shown are the percent ± SD values of dying DN, DP $CD25^{hi}$ and SP4 $CD25^{hi}$ T-reg precursors in one of three representative experiments. Strength of CD3/CD28 stimulation (X-axis) refers to the amount of antibodies per 10^6 cells in 0.2 ml medium.

Figure 5. Homeostatic Normalization of Splenic $CD4^{+}25^{hi}$ T-reg Compartment in BALB/c and B10.D2 mice

(A) Mice (n = 5) were injected with CFSE, and four and six days later the splenocytes were stained with CD4-PE-Cy7 and CD25-APC Ab-conjugates. The number of cell cycle divisions in FACS-gated $CD4^{+}25^{hi}$ thymocytes was established based on the dilution factor for CFSE fluorescence The percent of proliferating T-reg cells in each cycle of division was determined using the ModFit analysis software. Shown is one of five representative mice in each group. Empty histograms represent day 6, and dark

histograms represent day 4 post-CFSE injection. Arrow in the middle panel indicates the presence of a slow-dividing $CD4^{+}25^{hi}$ T-reg population in the spleen of a B10.D2 mouse. Lower panel shows the CFSE fluorescence after five serial, twofold dilutions *in vitro* (empty plots) as compared with the uptake of CFSE by thymocytes *in vivo* at 30 min after CFSE injection (dark plot).

(B) ObR partitioning on FACS-gated $CD4^{+}25^{hi}$ T-reg splenic cells from BALB/c and B10.D2 mice as determined by FACS using ObR-FITC Ab-conjugate (BD PharMingen). Same mice used for thymic ObR measurements as described in figure 2 (panel b) were used in this experiment. Shown are the MFI ± SD values (bold), and percent of $CD4^{+}CD25^{hi}$ splenocytes from individual mice (n = 4 per group) expressing low and high ObR density on the surface.

Figure 6. Differential Suppressogenicity of Splenic $CD4^{+}25^{hi}$ T-regs from BALB/c and B10.D2 mice

(A) *In vitro* suppression assay. The $CD4^{+}25^{-}$ T cell responders ($5x10^{5}$ cells) from the spleens of BALB/c or B10.D2 mice (pool of 5 mice per group) were isolated on CD4 mouse columns followed by CD25 immunobeads, and then co-incubated for 24 h on CD3 and CD28 Ab stimulation (2.5 μg/$5x10^{5}$ cells) with FACS-sorted, syngeneic $CD4^{+}25^{hi}$ T-reg cells ($5x10^{5}$ cells). From the same culture wells, the supernatants were assessed for cytokine secretion by ELISA, and the cell pellets were assessed for the mRNA expression level of Th1 and Th2 transcriptional factors by real-time PCR. Shown is the mean inhibition ± SD of IL-2, IL-4, and IFN-γ from two identical experiments in which the signal-to-noise signal generated in the T responders/T-reg co-culture in the absence of

CD3 and CD28 stimulation were subtracted (*upper panel*). The percent inhibition of cytokine secretion (Y-axis) was determined in relation to the corresponding amount of IL-2, IL-4 and IFN-γ (pg/ml) secreted by $CD4^{+}25^{-}$ T cell responders stimulated with CD3/CD28 Abs in the absence of T-reg cells. The quantitative measurements of mRNA relative expression for T-bet, STAT4, NFATc, STAT6, GATA3, and cMAF were carried out by real-time PCR (*lower panel*) (specific primers, Applied Biosystems, Foster City, CA). The percent inhibition for each transcription factor (Y-axis) was calculated as percent of mRNA obtained by real-time PCR from $CD4^{+}25^{-}$ T cell responders stimulated with CD3/CD28 Abs in the absence of T-reg cells.

(B) RAG2 KO, RIP-HA Tg mice infused with splenic T-reg cells (10^5 cells from a pool of five BALB/c or five B10.D2 mice) (arrow at day 0 on X-axis), and 7 days later infused with diabetogenic, splenic T cells ($2.5x10^5$ cells from a pool of five TCR-HA Tg mice) (arrow at day 7 on X-axis). Shown are the glycemia values (u*pper panel)* and the percent survival (l*ower panel)* of RAG2 KO, RIP-HA Tg recipients. The dotted line (*upper panel*) indicates the upper limit of normoglycemia previously determined in a cohort of twelve non-infused (control) RAG^{-2} RIP-HA Tg mice.

(C) Hematoxilin-eosin staining of pancreatic cross-sections from RAG2 KO, RIP-HA Tg recipients treated like in panel B, at 1 week after infusion of diabetogenic T-cells. Shown is a one of twenty representative pancreatic islets from each group of mice.

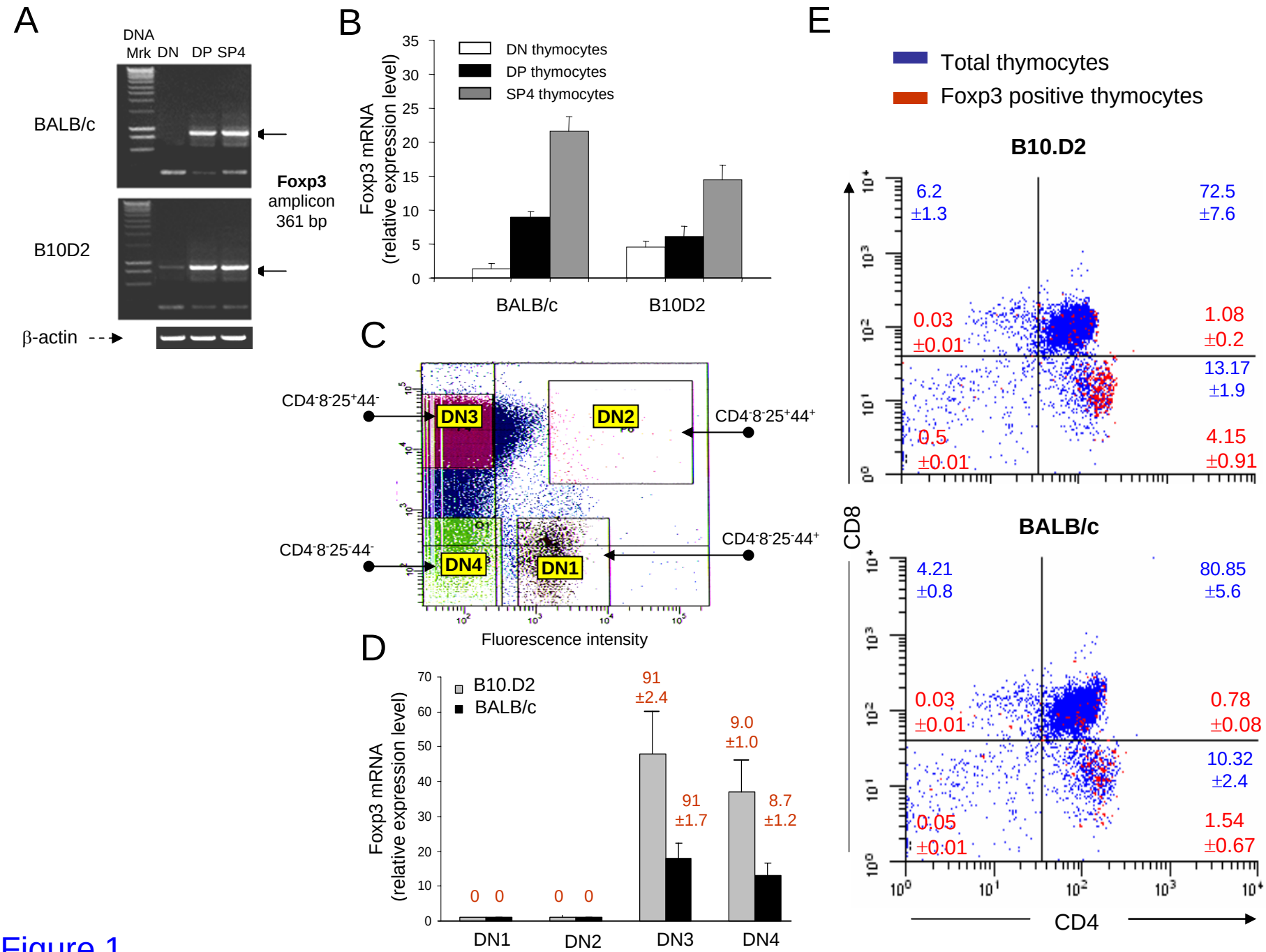

A
DNA
Mrk DN DP SP4
BALB/c
B10D2
Foxp3
amplicon
361 bp
β-actin
B
DN thymocytes
DP thymocytes
SP4 thymocytes
Foxp3 mRNA
(relative expression level)
BALB/c
B10D2
C
CD4-8-25+44-
CD4-8-25+44+
CD4-8-25-44-
CD4-8-25-44+
DN3
DN2
DN4
DN1
Fluorescence intensity
D
B10.D2
BALB/c
Foxp3 mRNA
(relative expression level)
91 ±2.4
91 ±1.7
9.0 ±1.0
8.7 ±1.2
0 0
0 0
DN1
DN2
DN3
DN4
E
Total thymocytes
Foxp3 positive thymocytes
B10.D2
6.2 ±1.3
72.5 ±7.6
0.03 ±0.01
1.08 ±0.2
13.17 ±1.9
0.5 ±0.01
4.15 ±0.91
BALB/c
4.21 ±0.8
80.85 ±5.6
0.03 ±0.01
0.78 ±0.08
10.32 ±2.4
0.05 ±0.01
1.54 ±0.67
CD8
CD4

Figure 1

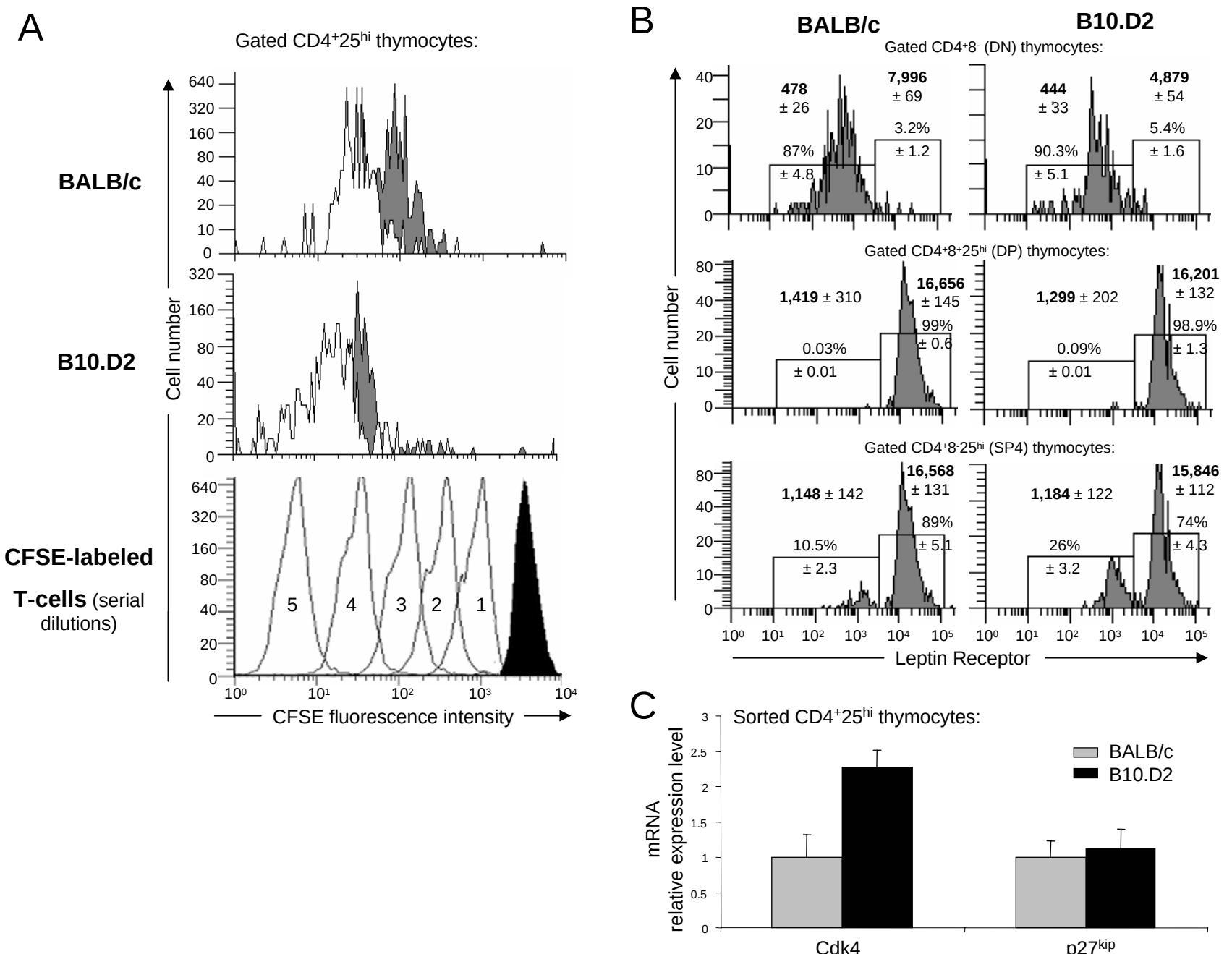
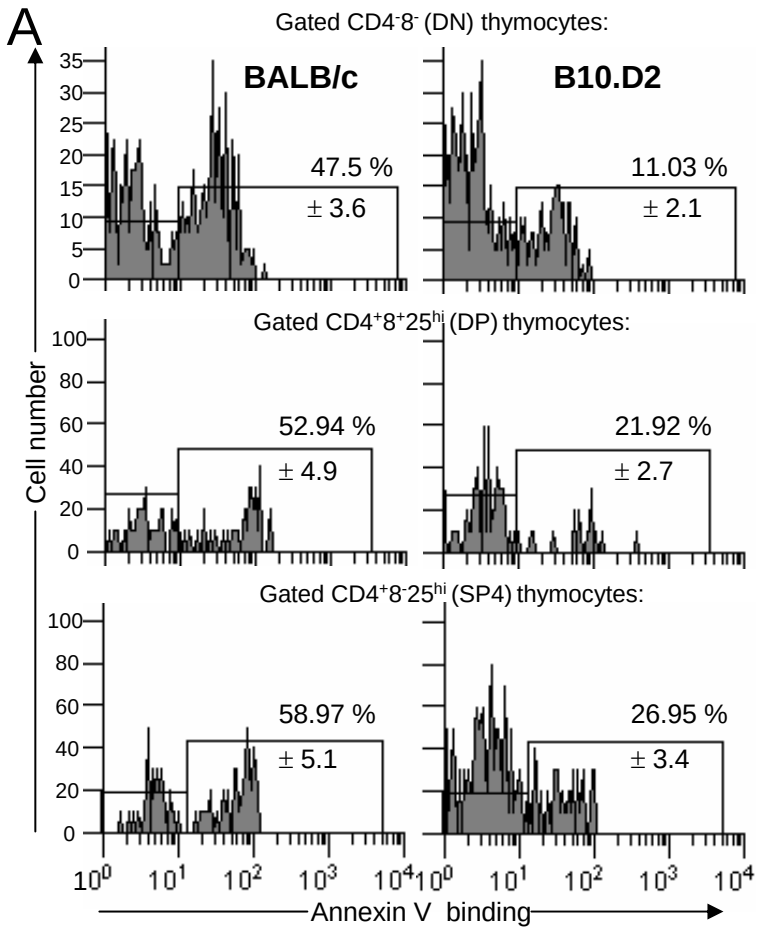
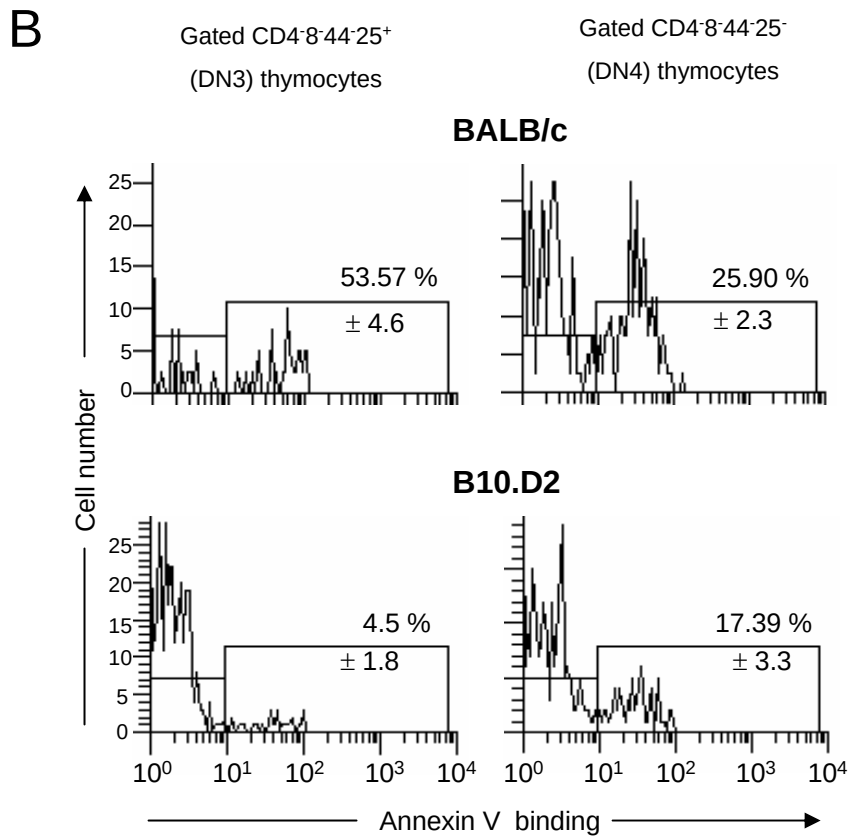

A
Gated CD4+25hi thymocytes:
BALB/c
B10.D2
CFSE-labeled
T-cells (serial dilutions)
Cell number
5
4
3
2
1
CFSE fluorescence intensity
B
BALB/c
B10.D2
Gated CD4+8- (DN) thymocytes:
478 ± 26
87% ± 4.8
7,996 ± 69
3.2% ± 1.2
444 ± 33
90.3% ± 5.1
4,879 ± 54
5.4% ± 1.6
Gated CD4+8+25hi (DP) thymocytes:
1,419 ± 310
0.03% ± 0.01
16,656 ± 145
99% ± 0.6
1,299 ± 202
0.09% ± 0.01
16,201 ± 132
98.9% ± 1.3
Gated CD4+8-25hi (SP4) thymocytes:
1,148 ± 142
10.5% ± 2.3
16,568 ± 131
89% ± 5.1
1,184 ± 122
26% ± 3.2
15,846 ± 112
74% ± 4.3
Cell number
Leptin Receptor
C
Sorted CD4+25hi thymocytes:
mRNA relative expression level
BALB/c
B10.D2
Cdk4
p27kip

Figure 2





C

Mouse:		Bcl-2	Bcl-X_L	Bad	Bax	Caspase 3	Fas	FasL
BALB/cj	mRNA(*)	2,815	1,114	**4,118**	864	**13,211**	5,105	2,008
	Protein(**)	124	86	**281**	72	ND	302	114
	Activity(***)	-	-	-	-	**6.3**	-	-
B10D2	mRNA	2,964	1,203	1,340	916	5,724	4,818	2,051
	Protein	136	87	99	79	ND	281	109
	Activity	-	-	-	-	2.7	-	-

FIGURE 3

A

Thymocytes	*CD3ε*		*CD28*	
	B10.D2	BALB/c	B10.D2	BALB/c
	Mean Fluorescence Intensity (MFI)			
$CD4^-8^-$ (**DN**)	67.60 ± 5.6	141.75 ±19.4	13.58 ± 2.3	12.41 ± 1.8
$CD4^+8^+25^{hi}$ (**DP**)	118.85 ± 15.5	472.87 ± 23.1	10.40 ± 3.1	10.19 ± 2.0
$CD4^+8^-25^{hi}$ (**SP4**)	296.65 ±14.7	309.84 ± 12.4	36.65 ± 3.7	30.41 ± 5.1

B

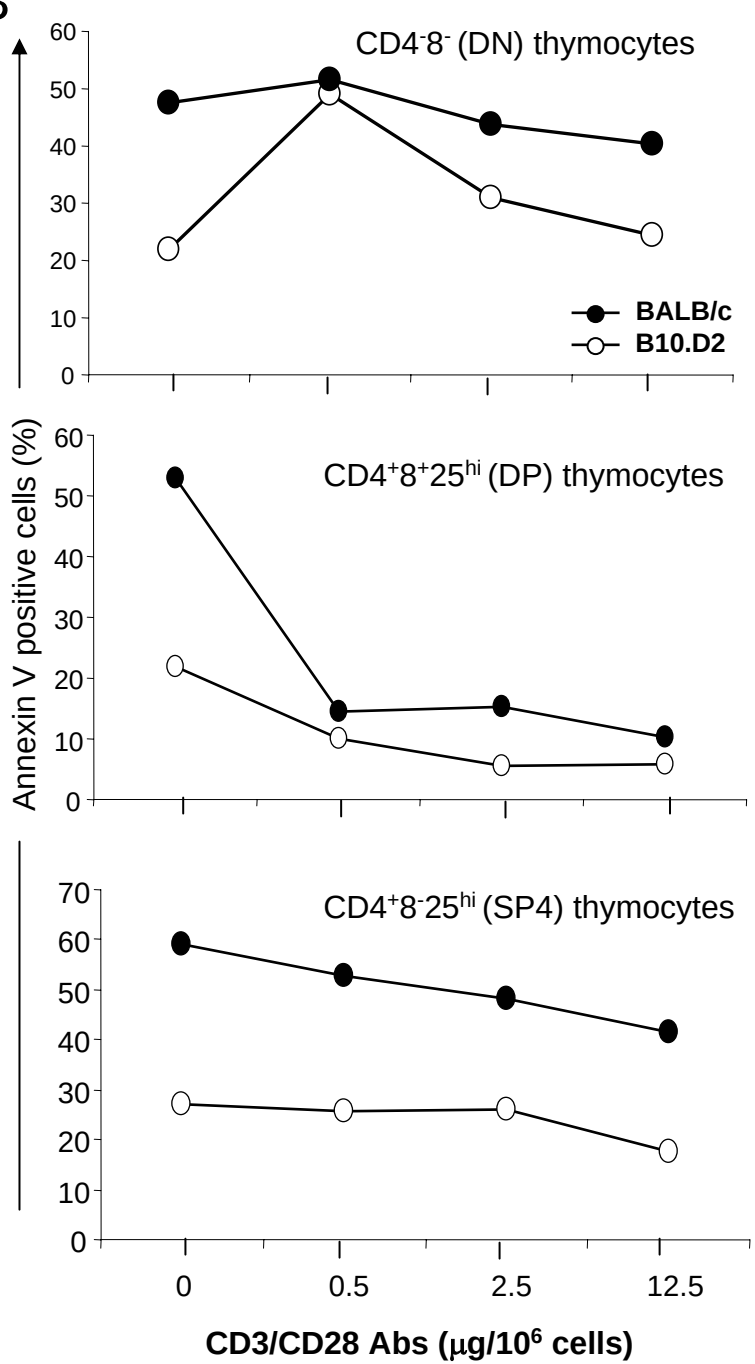


Figure 4

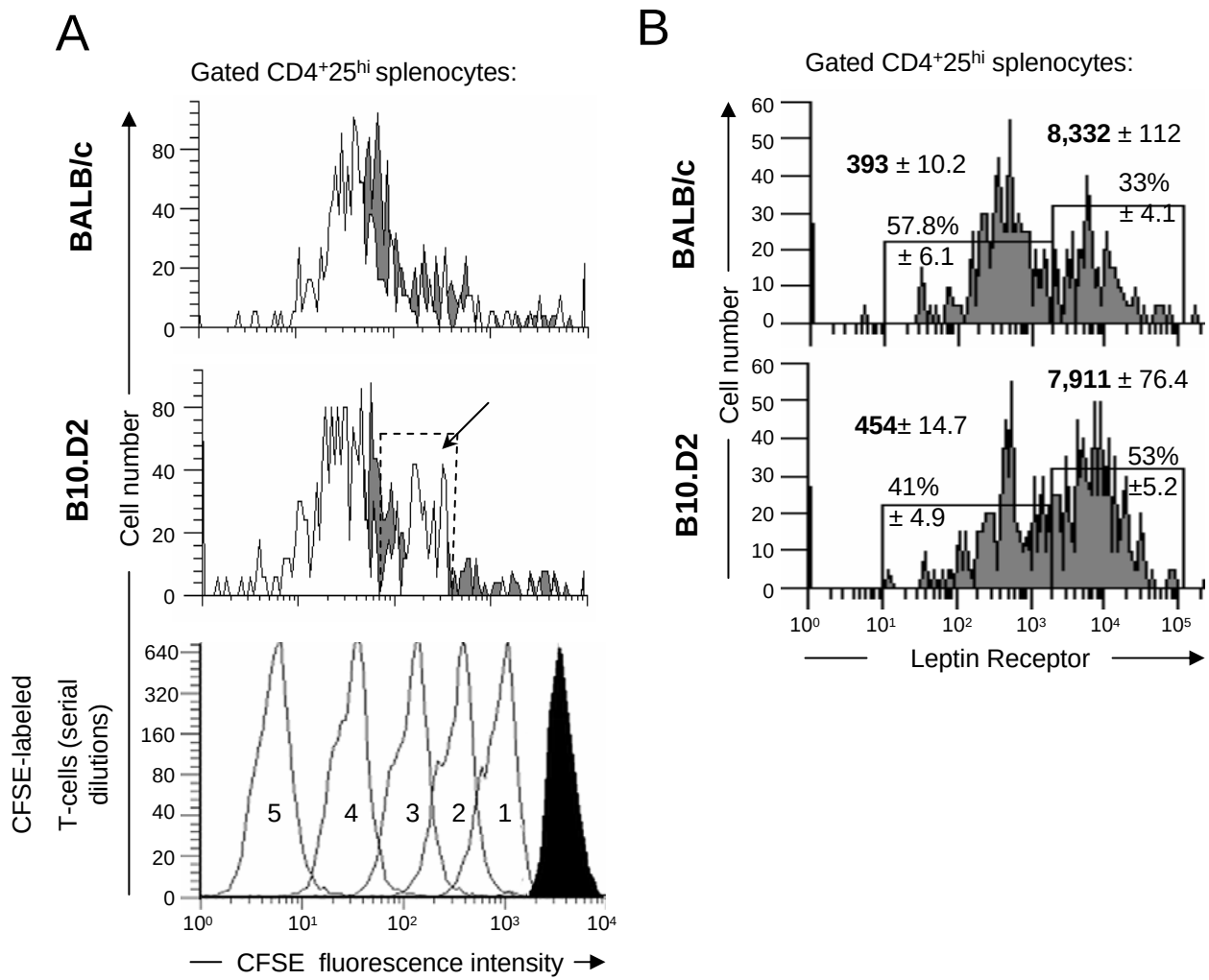

A
Gated CD4+25hi splenocytes:
BALB/c
B10.D2
Cell number
CFSE-labeled T-cells (serial dilutions)
5
4
3
2
1
CFSE fluorescence intensity
B
Gated CD4+25hi splenocytes:
BALB/c
B10.D2
Cell number
393 ± 10.2
57.8% ± 6.1
8,332 ± 112
33% ± 4.1
454± 14.7
41% ± 4.9
7,911 ± 76.4
53% ±5.2
Leptin Receptor

Figure 5

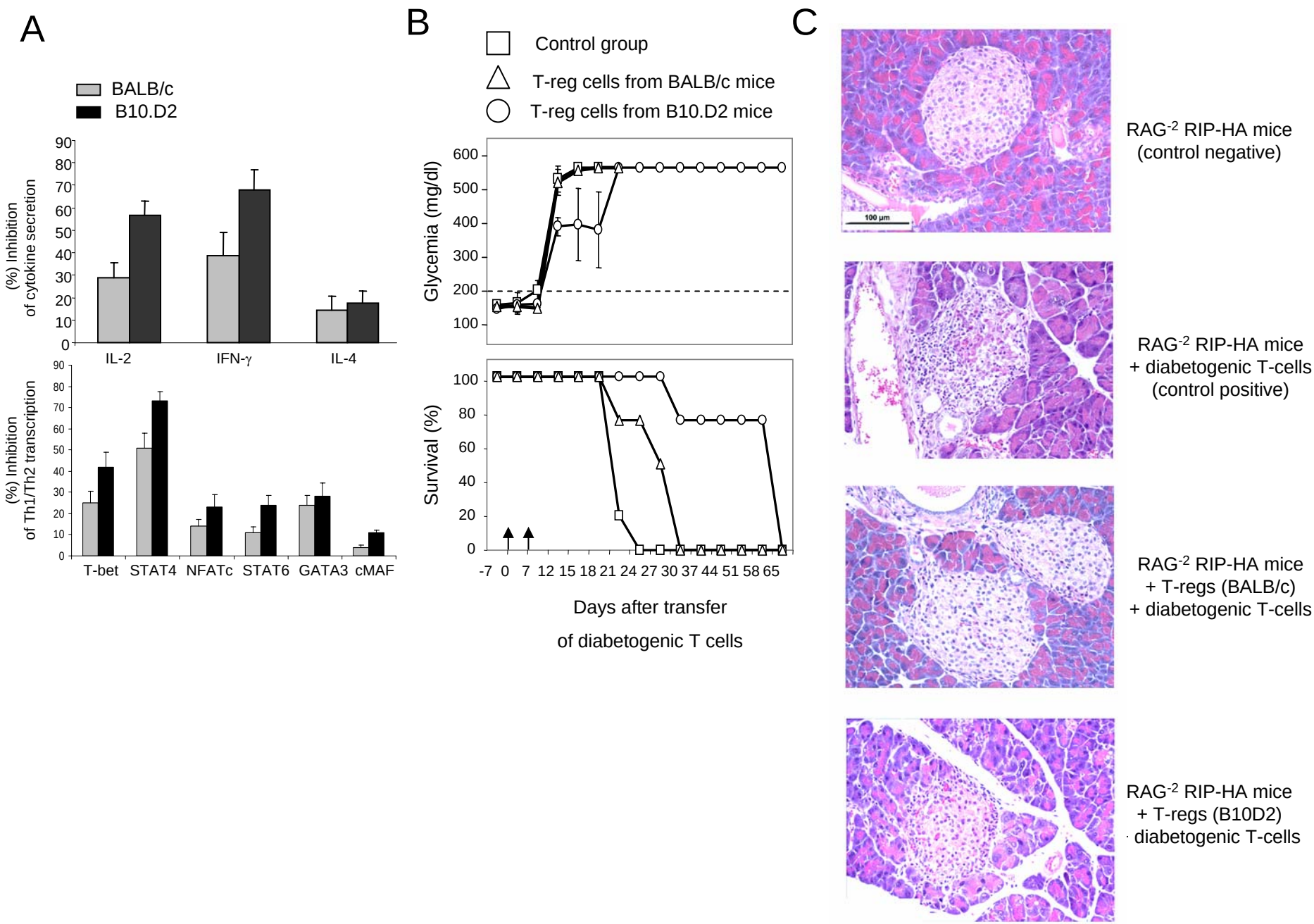

A
BALB/c
B10.D2
(%) Inhibition of cytokine secretion
IL-2
IFN-γ
IL-4
(%) Inhibition of Th1/Th2 transcription
T-bet
STAT4
NFATc
STAT6
GATA3
cMAF
B
Control group
T-reg cells from BALB/c mice
T-reg cells from B10.D2 mice
Glycemia (mg/dl)
Survival (%)
Days after transfer of diabetogenic T cells
C
RAG-2 RIP-HA mice (control negative)
RAG-2 RIP-HA mice + diabetogenic T-cells (control positive)
RAG-2 RIP-HA mice + T-regs (BALB/c) + diabetogenic T-cells
RAG-2 RIP-HA mice + T-regs (B10D2) · diabetogenic T-cells

Figure 6

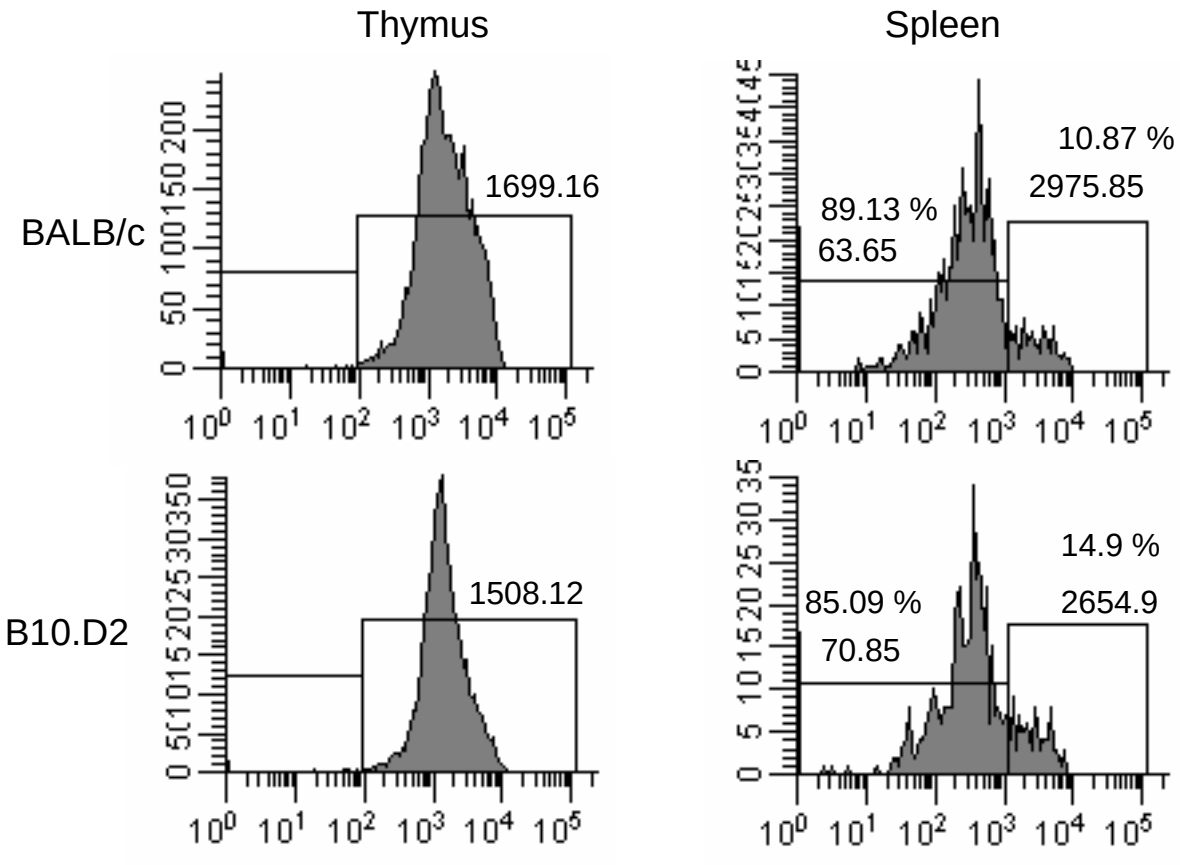

Thymus
Spleen
BALB/c
B10.D2
1699.16
89.13 %
63.65
10.87 %
2975.85
1508.12
85.09 %
70.85
14.9 %
2654.9

Figure S1

DNA methylation sites in the 1st and 2nd amplicons of Foxp3 gene from BALB/c and B10.D2 thymocytes

```
B10.D2  1    AGGCTGACATTCCAGAGCCAGCAAGAGGCCTTATGGAGTTTTAAGCTTCCTGGCTTTAGGTGGTTCCCATTTCTT  75
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       AGGCTGACATTCCAGAGCCAGCAAGAGGCCTTATGGAGTTTTAAGCTTCCTGGCTTTAGGTGGTTCCCATTTCTT

B10.D2  76   TGGGCTCTGGGACATCAATACACACAGTAAGAAGGTGGATCCATGCACCCTACAGAGTCTGTGTTCTTGAGATTC 150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       TGGGCTCTGGGACATCAATACACACAGTAAGAAGGTGGATCCATGCACCCTACAGAGTCTGTGTTCTTGAGATTC

B10.D2  151  TAAAATCCGTTGGCTTTGAGAAATGATATCGTACAGTTCTGAGTTTCTGTTACTACAGCATTTGAAGACTCAAGG 225
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       TAAAATCCGTTGGCTTTGAGAAATGATATCGTACAGTTCTGAGTTTCTGTTACTACAGCATTTGAAGACTCAAGG

B10.D2  226  GGGTCTCAATATCCATGAGGCCTGCCTAATACTCACCAAGCATCCAACCTTGGGCCCCTCTGGCATCCAAGAAAG 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       GGGTCTCAATATCCATGAGGCCTGCCTAATACTCACCAAGCATCCAACCTTGGGCCCCTCTGGCATCCAAGAAAG

B10.D2  301  ACAGAATCGATAGAACTTGGGTTTTGCATGGTAGCCAGATGGACGTCACCTACCACATCCGCTAGCACCCACATC 375
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       ACAGAATCGATAGAACTTGGGTTTTGCATGGTAGCCAGATGGACGTCACCTACCACATCCGCTAGCACCCACATC

B10.D2  376  ACCCTACCTGGGCCTATCCGGCTACAGGATAGACTAGCCACTTCTCGGAACGAAACCTGTGGGGTAGATTATCTG 450
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       ACCCTACCTGGGCCTATCCGGCTACAGGATAGACTAGCCACTTCTCGGAACGAAACCTGTGGGGTAGATTATCTG

B10.D2  451  CCCCCTTCTCTTCCTCCTTGTTGCCGATGAAGCCCAATGCATCCGGCCGCCATGACGTCAATGGCAGAAAAATCT 525
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       CCCCCTTCTCTTCCTCCTTGTTGCCGATGAAGCCCAATGCATCCGGCCGCCATGACGTCAATGGCAGAAAAATCT

B10.D2  526  GGCCAAGTTCAGGTTGTGACAACAGGGCCCAGATGTAGACCCCGATAGGAAAACATATTCTATGTCCCAGAAACA 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       GGCCAAGTTCAGGTTGTGACAACAGGGCCCAGATGTAGACCCCGATAGGAAAACATATTCTATGTCCCAGAAACA

B10.D2  601  ACCTCCATACAGCTTCTAAGAAACAGTCAAACAGGAACGCCCCAACAGACAGTGCAGGAAGCTGGCTGGCCAGCC 675
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       ACCTCCATACAGCTTCTAAGAAACAGTCAAACAGGAACGCCCCAACAGACAGTGCAGGAAGCTGGCTGGCCAGCC

B10.D2  676  CAGCCCTCCAGGTCCCTAGTACCACTAGACAGACCATATCCAATTCAGG  724
             |||||||||||||||||||||||||||||||||||||||||||||||
BALB/c       CAGCCCTCCAGGTCCCTAGTACCACTAGACAGACCATATCCAATTCAGG
```

Figure S1

MANUSCRIPT 2:

CD28 Signaling Requires Rafts Integrity and a p56^{lck} Motif to Stabilize Foxp3 transcript in CD4^{+}25^{hi} T Regulatory Precursors

By:

Cristina Stoica[1], Constantin Bona[2], Sofia Casares[1,3], and Teodor-D. Brumeanu[1,4]

From:

[1] Department of Medicine, Division of Immunology, Uniformed Services University of Health Sciences, Bethesda, Maryland 20814, U.S.A;

[2] Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029, U.S.A;

[3] Naval Medical Research Center, Infectious Diseases Directorate, Division of Malaria, Silver Spring, Maryland 20910, U.S.A.

Keywords: CD28, Foxp3, p56^{lck}, Lipid rafts, T regulatory cells.

[4] Corresponding author: Dr. T-D. Brumeanu, The Uniformed Services University of Health Sciences, Department of Medicine, Division of Immunology, 4301 Jones Bridge Road, Bethesda, MD 20814, Email address: tbrumeanu@usuhs.mil.

[5] This study was supported by grants from the National Institutes of Health, DK61927 & DK61326 to T-D.B. and DK077521 to S.C, and JDRF-12002-1151 to SC and T-D.B.

[6] Abbreviations used in this paper: CFSE, Carboxy Fluoroscein Succinimidyl Ester; CTX-B, Cholera Toxin B subunit; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimide-azole; MβCD, Methyl-β-cyclodextrin; MFI, Mean Fluorescence Intensity; TcH, T-cell Hybridoma; T-reg, T regulatory cell; wt, wild type.

Abstract

T regulatory cells (T-regs) are critical for maintaining tolerance to self and non-self. Naturally occurring $CD4^{+}25^{hi}$ T-regs in thymus express the Foxp3 master-regulatory gene responsible for their development and suppressogenicity. Herein, we found that CD28 costimulation alone augmented, but did not induce de novo Foxp3 expression in T-reg thymic precursors. The CD28-upregulation of Foxp3 relied on mRNA stabilization, and required plasma membrane rafts integrity as well as a functional $p56^{lck}$ binding motif on the CD28 cytosolic tail. Noteworthy, the glycosphingolipids and cholesterol rafts components were unusual partitioned in T-reg precursors as compared with conventional T-cell precursors. The CD28-upregulation of Foxp3 was paralleled by an increase in thymic proliferation and in suppressogenicity of terminally differentiated $CD4^{+}25^{hi}$ T-reg thymocytes. Extending the span-life of Foxp3 transcripts in a rafts/$p56^{lck}$ manner represents a novel mechanism by which CD28 co-receptor fosters the development and suppressogenicity of $CD4^{+}25^{hi}$ T-reg precursors in thymus.

The "professional" $CD4^{+}25^{hi}$ regulatory T cells (T-regs) represent a subset of T regulatory lymphocytes naturally born in the thymus (1). T-regs are the "guardians" of the immune system, as they maintain homeostasis of T-cell compartment and at the same time restrict conventional CD4 and CD8 T-cell responses to *self* and non-*self* antigens (2-5). The $CD4^{+}25^{hi}$ T-reg cells express preferentially the *Foxp3* protein (Scurfin), a forkhead-winged helix transcription factor (6). There is now compelling evidence that Foxp3 is the master gene switch for the thymic development and function of $CD4^{+}25^{hi}$ T-regulatory cells (7-8).

CD28 costimulation enhances the CD3/TCR signaling of T-cells toward growth, proliferation, and survival by up-regulation of IL-2 and Bcl-XL mRNA expression at the transcriptional and pot-transcriptional levels (9-14), and it also strengthens the APC-T-cell immunological synapse (15-16). It has been previously shown that CD28 co-stimulation helps initiation of thymic differentiation towards a Foxp3-expressing T-regulatory cell lineage through a signaling mechanism that requires a $p56^{lck}$ motif on the CD28 cytosolic tail also involved in IL-2 production (17). At the same time, the plasma membrane lipid rafts are able to recruit the CD28 co-receptor in the APC-T-cell immunological synapse to provide a proficient costimulation (18). However, the role of lipid rafts in signaling of T-regulatory cells is still unknown.

Herein, we provide evidence for a new mechanism by which CD28 costimulation solely up-regulates the Foxp3 expression in T-reg thymic precursors through stabilization of Foxp3 mRNA transcript. The $p56^{lck}$ binding motif on the cytosolic CD28 tail, but not the CD28-mediated PI-3K activation was critical for stabilization of Foxp3 mRNA transcript. We also show that the CD28 costimulation alone fosters the cell cycle division

of T-reg precursors in thymus, and enhances the suppressogenic capacity of terminally differentiated $CD4^{+}25^{hi}$ $Foxp3^{+}$ T-reg thymocytes. These signaling events were strictly depending on the integrity of plasma membrane lipid rafts on T-reg precursors.

Materials and methods

Mice

One month-old, recombinant inbred B10.D2 and CD28 Knockout (KO) mice on a C57BL/6 background were purchased from Jackson Laboratories. The CD28-CP mice are CD28 KO mice on C57BL/6 background and carrying a CD28 transgene mutated for $p56^{lck}$ motif on the cytosolic tail of CD28 co-receptor. The CD28-CP mice were kindly provided by Dr. A. Singer. Mice were housed at USUHS in a pathogen-free facility according to the federal and local regulations as reviewed/approved by the Uniformed Services University Institutional Review Committee.

Cell purification

The $CD4^{+}$ thymocytes and splenocytes from pools of mice (n = 6) or individual mice were negatively sorted on mouse CD4-enrichment columns (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. A second passage of $CD4^{+}$ cells on mouse CD8-enrichment columns (R & D Systems) yielded 90% pure and viable preparations of $CD4^{-}8^{-}$ double negative cells as determined by FACS and trypan blue exclusion method, respectively. The $CD4^{+}25^{+}$ thymocytes and splenocytes were isolated on MS MACS paramagnetic columns (Miltenyi Biotech Inc., Auburn, CA).

Briefly, the negatively-sorted $CD4^+8^-$ single positive or $CD4^-8^-$ double negative cells were incubated with anti-CD25-PE Ab-dye conjugate for 10 min, washed, and further incubated anti-PE Ab-microbeads for 15 min. The cells were sorted on magnetic columns (Miltenyi Biotec) into a $CD25^-$ fraction (flow through) and used in assays as T-cell responders, and into a $CD25^+$ fraction as T-reg cells (bound fraction).

RT-PCR and Quantitative Real-time RT- PCR

Total RNA and cDNA was prepared using NucleoSpin RNA II kit (BD Biosciences Clontech, Palo Alto, CA) and Qiagen One Step RT-PCR kit (Qiagen Inc., Valencia, CA), respectively, as previously described (19). Four nanograms of total RNA were required to prepare first-strand cDNA using a Qiagen One Step RT-PCR kit (Qiagen) at 35 and 40 cycles of amplification following the manufacturer's protocol. 25μL of each PCR amplicon was electrophoresed in 1.5% agarose gel containing ethidium bromide, and the percent of transcripts was calculated by Scion Image software analysis (Scion Corp., Frederick, MD) based on the integration of the percent of pixels and band intensity after normalization against the corresponding β-actin band, as we previously described (19). To measure the expression level of Foxp3 by quantitative real–time RT-PCR, total RNA was extracted from mouse lymphocytes using Total RNA Purification System (Invitrogen, Carlsbad, CA). Some 200 ng RNA was used to synthesize the first cDNA strand with the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR amplification mixtures contained Applied Biosystems Universal PCR MasterMix Buffer (25μl), cDNA template (22.5μl), labeled primers (2.5μl), respectively. Quantitative RT-PCR measurements were performed on an ABI Prism 7700 with SDS

1.9.1 Software (Applied Biosystems). The melting-curve analysis showed the specificity of the amplifications. The relative mRNA levels were estimated by the standard method using 18S rRNA as the reference gene. The specific primers for RT-PCR were: Foxp3, forward: CAGCTGCCTACAGTGCCCCTAG, and reverse: CATTTGCCAGCAGTGGG TAG) (19). Specific validated primers for Foxp3, T-bet, Stat4, and c-myc were from Applied Biosystems.

Flow cytofluorimetry

Thymocytes or splenocytes ($2x10^5$) were surface, intra-cellularly, or intra-nuclearly stained with various antibody-dye conjugates or their isotype controls (BD PharMingen, San Diego, CA). Negatively sorted $CD4^+$ T-cells were stained with Chorera Toxin B subunit (CTX-B)-FITC conjugate to measure the cell density of plasma membrane GM glycosphingolipids, (mean fluorescence intensity, MFI), or with Filipin III to measure the cell density of cholesterol in the plasma membrane as we previously described (20). Some 2,000 to 500,000 cell events were acquired on a LSR II Bekton-Dikinson instrument for each preparation. For accurate measurements of MFI values at the single-cell level, the slight difference in the size and signal-to-noise autofluorescence of CD4 splenocytes were compensated with the WINlist analysis software 3D5.0 (Verity Software House, Topsham, ME) during the data acquisition by FACS. Measured parameters were compared under various experimental conditions using the Sigmaplot analysis software v.8 (Aldrich-Sigma). Overall data were presented, for convenience, as mean ± SD of pooled samples.

mRNA decay assay

Purified $CD4^{+}25^{+}$ T-reg precursors were stimulated or not with 5μg CD28 mAb overnight, and then treated with 100 μM of DRB reagent (5,6-dichloro-1-β-D-ribofuranosyl-benzimide-azole) to stop *de novo* mRNA synthesis. Some 2 and 8 h post-DRB treatment, the cells (1x 10^6 cells) were washed and the pre-DRB treatment RNA was extracted using the Qiagen One Step RT-PCR kit (Qiagen Inc). Real time PCR was further carried out to measure the amount of Foxp3 and c-myc (control half-life) transcripts.

T reg suppression assay

Purified $CD4^{+}25^{hi}$ T reg precursors (10^6 cells) were stimulated or not with 5μg/ml CD28 mAb (clone #7D4, American Tissue Cell Cultures, Manassas, VA) overnight and co-cultured at 1:1 ratio with Th1 hybridoma responder cells (TcH 16-2-6). The TcH IL-2 secretion in cell culture supernatants was measured by ELISA kits (Biosource), and the Th1 transcription factors T-bet and STAT4 in TcH were measured by real-time PCR using specific primers from Applied Biosystems. The TcH does not secrete IL-4 and IL-10 spontaneously or upon stimulation with CD3 or CD28 Abs.

Lipid rafts disruption

Negatively sorted $CD4^{+}$ T-cells were treated with 5 mM of MβCD detergent (Methyl-beta-cyclodextrin, Sigma-Aldrich) for 1h, and stimulated overnight with CD28 mAb (5 μg/10^6 cells). Total RNA was extracted on the next day and real-time RT-PCR was performed to measure the amount of Foxp3 transcript using the 18S rRNA as endogenous

control. In parallel, cells treated or not with MβCD and CD28 mAb were stained with CD3- and CD25-dye conjugates, and with CTX-FITC or Filipin III to determine by FACS the amount and distribution of GM1 and cholesterol components of the lipid rafts in plasma membrane.

Results and Discussion

CD28 costimulation alone up-regulates Foxp3 expression, but does not induce de novo Foxp3 synthesis in T-reg precursors

It has been recently reported that CD28 costimulation alone augments the Foxp3 mRNA expression in T-reg precursors during their thymic development (17). In our experiments, the CD28 knock out (KO) mice on a C57Bl/6 background (B10.D2/CD28 KO) showed a significantly lower Foxp3 mRNA expression in all stages of thymic T-reg differentiation, i.e. $CD4^-8^-$ double negative (DN), $CD4^+8^+$ double positive (DP) and $CD4^+8^-$ single positive (SP4) thymocytes, as compared with the B10.D2/$CD28^{+/+}$ wild type (wt) strain (Fig. 1A). Since the absence of a functional CD28 receptor did not completely abrogate Foxp3 expression in T-reg precursors from B10.D2/CD28 KO mice, other signaling pathways are likely to be required for a proficient Foxp3 expression. Although not entirely functional in T-reg cells (21), the CD3/TCR pathway seemed to provide a positive signaling in T-reg precursors as observed by a slight increase in Foxp3 expression on CD3 stimulation alone (Fig. 1B).

To determine whether CD28 costimulation alone can trigger *de novo* Foxp3 expression, we took advantage of our recent finding, namely that the DN thymocytes

from B10.D2 wt mice express Foxp3 early in the DN3/4, but not in the DN1/2 stages of thymic differentiation (Stoica *et al.*, manuscript submitted). Neither CD28, nor CD3 stimulation of DN1/2 thymocytes from B10D2 wt mice (lacking Foxp3 mRNA expression in DN1/2 stages) were able to induce *de novo* Foxp3 expression (Fig. 1B). In addition, the mature, splenic $CD4^{+}25^{-}$ T-cells expressing tiny amounts of Foxp3 but considerably lower than the $CD4^{+}25^{hi}$ T-reg cells, as well as the splenic, mature $CD4^{+}25^{hi}$ T-reg cells showed a two-fold increase in Foxp3 mRNA expression upon CD28 costimulation alone (Fig. 1C). This indicated that the CD28 co-receptor augmented the Foxp3 expression not only in mature $CD4^{+}25^{hi}$ T-reg cells, but also in conventional, mature $CD4^{+}25^{-}$ T-cells.

These experiments showed first, that CD28 costimulation alone up-regulates Foxp3 mRNA expression in T-reg thymic progenitors. Second, the CD28 costimulation alone was able to up-regulate the expression of a pre-existent Foxp3 transcript in both precursors and mature T-reg cells, but was unable to induce *de novo* Foxp3 mRNA expression in T-cell precursors lacking Foxp3, such as the DN1/2 thymocytes.

CD28-induced up-regulation of Foxp3 expression in T-reg precursors relies on Foxp3 $p56^{lck}$-dependent mRNA stabilization

A major mechanism by which CD28 costimulation improves the T-cell survival relies on stabilization of IL-2 mRNA expression (11). Herein, we compared the span life of Foxp3 transcript in B10.D2 wt thymocytes in the absence or presence of CD28 costimulation using the DRB-mediated inhibition of *de novo* mRNA synthesis followed by a real-time PCR time-chase assay for the Foxp3 mRNA decay. The span-life of c-myc

transcript is very short (~40 min), and it was used as an endogenous control for Foxp3 transcript span-life. As depicted in figure 2A, the Foxp3 mRNA span life in thymic T-reg precursors was close to 2 h, but it significantly increased upon CD28 costimulation. Since the previous experiments indicated the inability of CD28 costimulation to induce Foxp3 *de novo* synthesis, it is most likely that a major mechanism underlying the increased amount of Foxp3 mRNA in T-reg precursors upon CD28 stimulation alone relied on Foxp3 mRNA stabilization leading to a cumulative process that has been detected by real-time PCR as an increased amount of Foxp3 transcript.

A C-terminal proline motif (PxxP) on the CD28 cytosolic tail recruits the $p56^{lck}$ kinase and allows its phosphorylation (22). Recently, Tai and colleagues showed that this motif is also involved in IL-2 production and at the same time responsible for Foxp3 up-regulation in T-reg cells (17). We thus questioned whether the CD28 $p56^{lck}$ motif, or the CD28-mediated PI-3K activation is required for Foxp3 mRNA stabilization in T-reg precursors. The $Foxp3^{+}$ T-reg thymic precursors from C57Bl/6 mice lacking the CD28 $p56^{lck}$ motif (CD28-CP mice) stimulated with CD28 Ab did not show a prolongation of Foxp3 mRNA span life as those from B10.D2 (C57BL/6) wt mice (Fig. 2B). In contrast, inhibition of PI-3K activation by the LY294002 inhibitor in the T-reg precursors from B10.D2 (C57BL/6) wt mice did not affect the CD28-mediated Foxp3 mRNA stabilization, as determined by the DRB assay followed by real-time PCR chase-time measurements. As illustrated in figure 2B, the LY294002 inhibitor of PI-3K also reduced considerably the c-myc mRNA expression, since c-myc activation is a down-stream event of PI-3K signaling pathway. This clearly indicated an efficient inhibition of PI-3K by LY294002. These results demonstrated a new mechanism by which CD28 costimulation

extends the half-life of Foxp3 transcript in T-reg precursors relies on a functional $p56^{lck}$ motif on the CD28 cytosolic tail rather than on PI-3K signaling pathway.

Lipid rafts disruption in T-reg precursors precludes the CD28-mediated stabilization of Foxp3 mRNA

The plasma membrane lipid rafts represent the primordial signaling platform for T-cell activation, since the rafts recruit and bring in close proximity a large number of receptors on the T-cell membrane, among which is the CD28 co-receptor (23). The CD28 recruitment in the immunological synapse is critical for a proficient T-cell activation by APCs loaded with immunogenic peptides (18). Since our experiments indicated that CD28 co-stimulation alone provides positive signaling for Foxp3 expression by a mechanism of mRNA stabilization, we next addressed the role of lipid rafts on the CD28-mediated stabilization of Foxp3 mRNA in T-reg precursors. A first observation was that, in contrast to the conventional ($CD4^+25^-$) thymic T-cell precursors, the $CD4^+25^{hi}$ thymic T-reg precursors expressed a significantly higher amount of GM glycosphingolipids (resident moiety of the lipid rafts), and cholesterol (the major component of lipid rafts) (Fig. 3A). Secondly, we found that stimulation of T-reg precursors with CD28 Ab did not alter significantly the rafts partitioning on T-reg plasma membrane. This is consistent with a report demonstrating that accumulation of rafts at the T-cell/APC immunological synapse occurs independently of CD28 ligation (24). However, some reports described an increased amount of rafts in T-cells upon CD28 costimulation (25-26). The difference in results may refer first, to the fact that CD28-induced up-regulation of rafts was detected in conventional, resting $CD4^+25^-$ T-cells, but not in T-reg cells, implying that the

threshold of CD28 signaling in conventional T cells is different than that in T-reg cells. A second possibility is that the high amount of rafts in T-reg precursors as found in this study (as compared with conventional T-cell precursors) may not allow FACS detection of incremental rafts increases between CD28 stimulated and non stimulated T-reg cells.

Disruption of lipid rafts by MβCD in T-reg precursors affected drastically the GM1 and cholesterol partitioning in the plasma membrane (Fig. 3A), and precluded the CD28-mediated up-regulation of Foxp3 expression (Fig. 3B). These results clearly demonstrated that the lipid rafts integrity plays an essential role in the CD28-mediated up-regulation of Foxp3 expression in thymic T-reg precursors. Lipid rafts were previously shown to recruit $p56^{lck}$ at the inner face of the plasma membrane in conventional T-cells (27), which suggests that the $p56^{lck}$ binding motif required for Foxp3 mRNA stabilization may have been poorly recruited or activated on the CD28 cytosolic tail upon rafts disruption.

CD28-induced up-regulation of Foxp3 expression parallels the increase in rate of thymic proliferations and suppressogenicity of T-reg precursors

It has been shown that CD28 signaling is required for a proficient generation of T-reg cells in thymus (17). To find out whether CD28 costimulation alone can affect the rate of T-reg thymic proliferation, CSFE-labeled thymocytes from B10D2 wt mice were stimulated *in vitro* with CD28 mAb, and the number of cell divisions were estimated in relation to the extend of CSFE dilution at the single-cell level by FACS. In line with previous observations (17), we found that CD28 stimulation solely augmented the cell cycle progression of $CD4^{+}25^{hi}$ thymocytes, and it also increased significantly their

survival *in vitro* (Fig. 4A). Only 5 to 10% of $CD4^{+}25^{hi}$ thymocytes stimulated with CD28 Ab entered apoptosis at the end of 3rd day as determined by Annexin V binding in FACS (data not shown). The rate of proliferation of $CD4^{+}25^{hi}$ thymocytes *in vitro* upon CD28 costimulation alone was pretty much similar to that observed *in vivo*, i.e. 3 cycles of division within 3 days (Stoica et al., manuscript submitted). These results indicated that CD28 costimulation alone provides positive signals for proliferation and survival of T-reg precursors.

It has been also shown that, likewise mature T-reg cells, the $CD4^{+}8^{-}25^{hi}$ T-reg thymocytes can suppress other conventional $CD4^{+}25^{-}$ T-cells (28). Herein, we employed a new *in vitro* system to investigate whether the CD28 costimulation alone could affect the suppressogenic capacity of terminally differentiated $CD4^{+}8^{-}25^{hi}$ T-reg thymocytes. For this, the IL-2 secretion and Th1 transcriptional activity of TcH 16-2-6 that secretes spontaneously IL-2 was measured upon co-culturing with terminally differentiated $CD4^{+}25^{hi}$ T-reg precursors in the presence or not of CD28 mAb. Of note, the CD28 costimulation does not alter the TcH growth or its IL-2 secretion, or its rate of proliferation as determined by the number of CSFE-labeled TcH and number of cell cycle divisions (data not shown). In contrast, the IL-2 secretion by TcH was inhibited by 30-40% in CD28-stimulated $CD4^{+}(8^{-})25^{hi}$ T-reg thymocytes (Fig. 4B), and T-bet mRNA, but not STAT4 mRNA expression in TcH was reduced 5-times as compared with non stimulated $CD4^{+}(8^{-})25^{hi}$ T-reg cultures (Fig. 4C). This clearly indicated that stimulation of terminally differentiated $CD4^{+}8^{-}25^{hi}$ T-reg thymocytes with CD28 Ab alone can augment their suppressogenic capacity. The CD28-mediated increase in T-reg suppressogenicity occurred simultaneously with up-regulation of Foxp3, as we previously

described in this study (Fig. 2). The mechanism by which Foxp3 confers suppressogenicity to T-reg cells is unknown. Nonetheless, a strong correlation between the suppressogenic capacity and amount of Foxp3 protein in T-reg cells as found in this study, was also reported (29).

In summary, in this study we showed for the first time that CD28 costimulation alone augments the development and suppressogenicity of $CD4^{+}25^{hi}$ T-reg precursors in thymus by a mechanism of Foxp3 mRNA stabilization, and that a functional $p56^{lck}$ binding motif on the CD28 cytosolic tail as well as the integrity of lipid rafts are essential for this mechanism to operate properly.

Acknowledgements: We thank Mrs Karen Wolcott and Kateryna Lund for technical assistance with FACS sorting of T-reg precursors, and Dr. Alfred Singer (NIH/NCI) for providing the CD28-CP mice.

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Figure legends

Figure 1. Foxp3 regulation in T-reg precursors by CD28 costimulation. (A) The relative expression levels of Foxp3 mRNA (Y-axis) in double negative ($CD4^-8^-$, DN), double positive ($CD4^+8^+$, DP), and single positive ($CD4^+8^-$, SP4) thymocytes from B10.D2 wt and B10.D2/CD28 KO mice were calculated in relation to the lowest level of Foxp3 mRNA as found in the DN thymocytes of B10D2 CD28 KO mice. Shown are the mean values ± SD from five individual mice in each group. (B) FACS-sorted DN1-4 thymocytes were incubated for 24 h with nil (*lane 1*), or 5 μg/10^6 cells of CD3ε (2C11) *(lane 2*) or CD28 (7D4) mAb (*lane 3*) and analyzed by RT-PCR for the level of Foxp3 mRNA expression (Y axis). Shown are the Foxp3 transcripts at 40 cycles of PCR amplification for one of four representative B10.D2 wt mice. (C) Conventional, splenic $CD4^+25^-$ T-cells and $CD4^+25^+$ thymocytes from B10.D2 wt mice were stimulated or not with CD28 mAb (5 μg/10^6 cells) for 24 h, and the relative level of Foxp3 mRNA expression was measured by real-time PCR. Shown are the mean values ± SD from five individual mice.

Figure 2. Foxp3 mRNA decay in T-reg precursors on CD28 costimulation. (A) The $CD4^+25^+$ thymocytes from B10.D2 wt mice on CD28 stimulation for 24 h were treated or not with DRB, and the mRNA fold increase for Foxp3 and c-myc genes (Y axis) was measured by real-time PCR as described. Shown are the mean values ± SD from four individual mice. (B) Same experiment as described in panel A was carried out in $CD4^+25^+$ thymocytes from B10.D2 wt mice on incubation with a specific PI-3K inhibitor

(LY294002) and in B10.D2 CD28-CP mice carrying a mutated, non functional $p56^{lck}$ binding motif on CD28 cytosolic tail. Shown are the mean values ± SD of two experiments.

Figure 3. Relation between the lipid rafts integrity and Foxp3 expression in T-reg precursors upon CD28 costimulation. (A) Negatively-sorted CD4 thymocytes from B10.D2 wt mice were first treated with NIl, CD28 Ab, CD28 Ab plus β-MCD, or β-MCD alone, and then washed and stained with a combination of CD25-PerCP-Cy5.5 and CTX-B-FITC conjugates or Filipin III. Gated CD4+25- conventional T-cells precursors and CD4+25hi T-reg precursors were analyzed at the single-cell level by FACS for the partitioning patterns and amount of GM1 and cholesterol (MFI) in plasma membrane. Shown are the MFI values ± SD of three separate experiments. (B) Aliquot cells prepared as in panel A were analyzed for the relative expression level of Foxp3 mRNA by real-time PCR. Shown are the mRNA fold increase values for Foxp3 ± SD (Y axis) corresponding to three separate experiments.

Figure 4. Thymic growth regulation and suppressogenicity of T-reg precursors by CD28 costimulation. (A) Thymic proliferation of $CD4^{+}25^{hi}$ thymocytes on CD28 costimulation. *Upper panel*, total thymocytes from B10.D2 wt mice were labeled with CSFE, and then continuously stimulated with CD28 mAb (5 μg/10^6 cells) for 72 h, washed, and stained with CD4-APC-Cy7 and CD25-PerCP-Cy5.5. FACS gated $CD4^{+}25^{hi}$ thymocytes were analyzed for the number of cell cycle divisions based on the CSFE dilution factor in relation with a control made of two-fold serial dilutions of CSFE up taken by thymocytes

as in *lower panel.* Of note, a control of growth of $CD4^{+}25^{hi}$ thymocytes for three days in culture in the absence of CD28 costimulation is not included, as the cells do not grow, but they rather enter apoptosis and die in the absence of CD28 costimulation. (B) Inhibition of IL-2 secretion by TcH upon incubation with CD28-stimulated and non-stimulated $CD4^{+}25^{+}$ thymocytes from B10.D2 wt mice. The $CD4^{+}25^{+}$ thymocytes (T-reg precursors) were co-cultured for 2 days at 1:1 ratio with TcH responder cells, and the IL-2 secretion in cell culture supernatants was measured by ELISA kits. Shown are IL-2 mean values ± SD (Y axis) corresponding to three separate experiments. (C) Suppression of Th1 transcription factors in TcH upon incubation of CD28-stimulated and non-stimulated $CD4^{+}25^{+}$ thymocytes from B10.D2 wt mice. The $CD4^{+}25^{+}$ thymocytes (T-reg precursors) were co-cultured for 1 day at 1:1 ratio with TcH responder cells, and the T-bet and STAT4 mRNA relative expression levels were determined by real-time PCR. Dark bars correspond to cultures of TcH alone, white bars, the TcH co-cultured with T-reg precursors, and grey bars, the TcH co-cultured with T-reg precursors stimulated with CD28 Ab. Shown are the mean values of T-bet and STAT4 mRNA expression levels ± SD (Y axis) corresponding to three separate experiments.

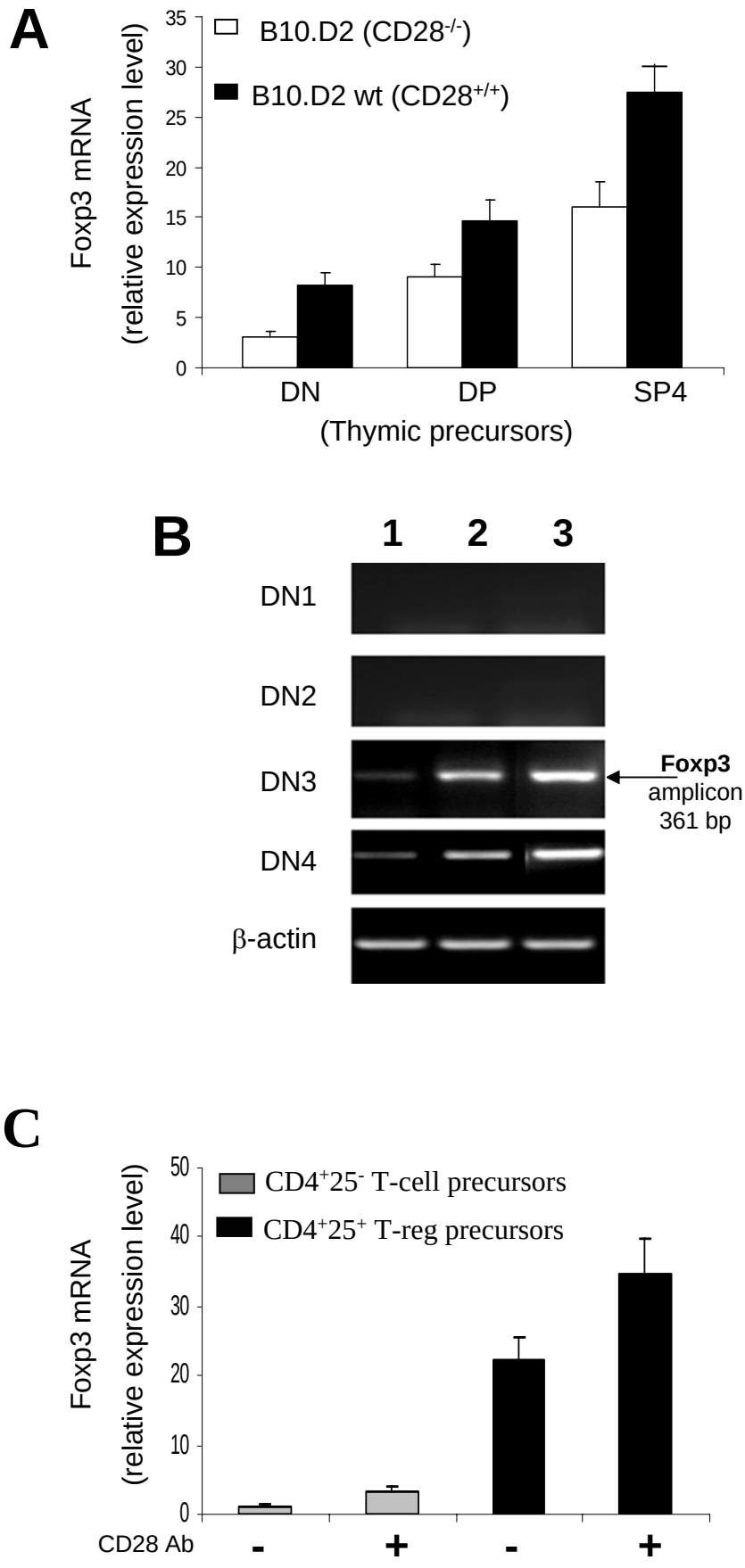

A
B10.D2 (CD28-/-)
B10.D2 wt (CD28+/+)
Foxp3 mRNA
(relative expression level)
35
30
25
20
15
10
5
0
DN
DP
SP4
(Thymic precursors)
B
1
2
3
DN1
DN2
DN3
DN4
β-actin
Foxp3
amplicon
361 bp
C
CD4+25- T-cell precursors
CD4+25+ T-reg precursors
Foxp3 mRNA
(relative expression level)
50
40
30
20
10
0
CD28 Ab
-
+
-
+

Figure 1

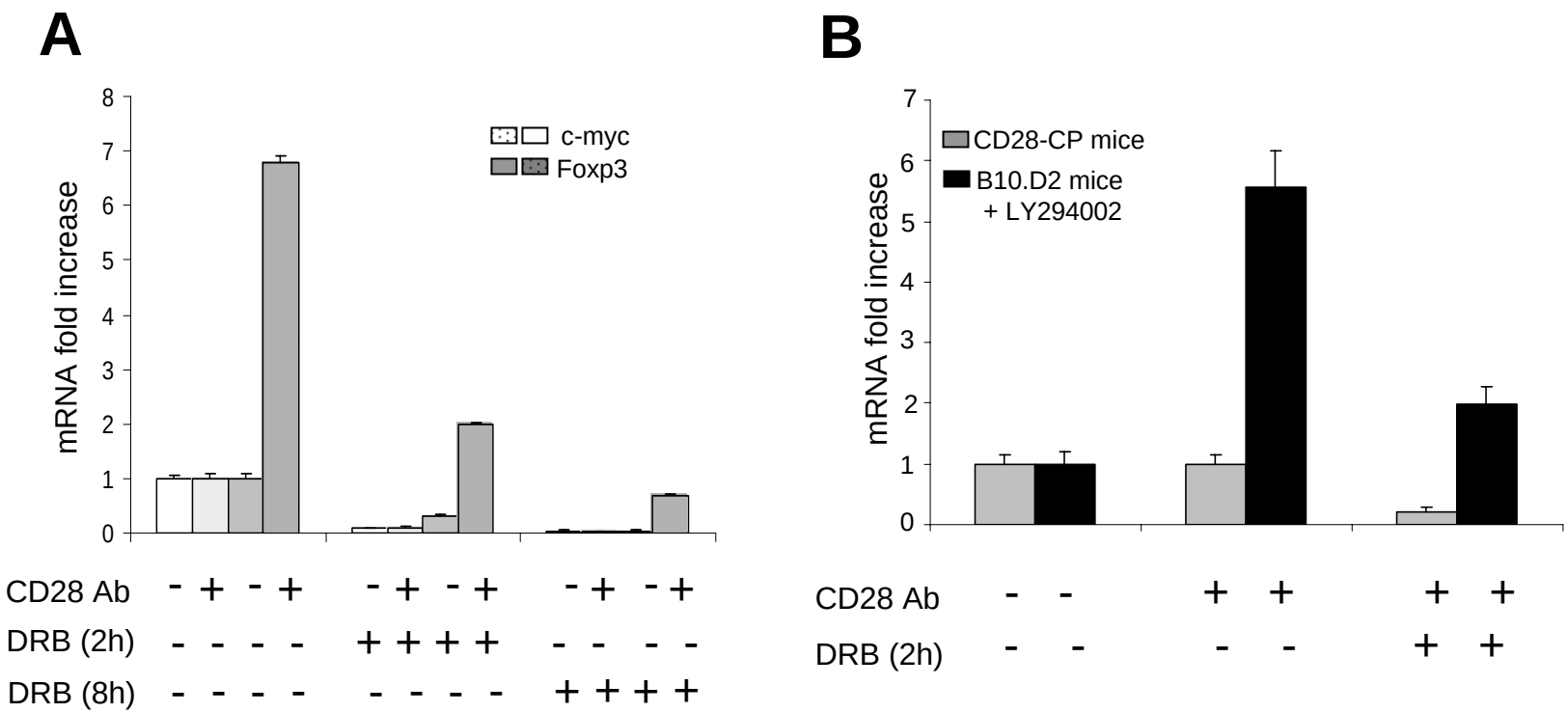
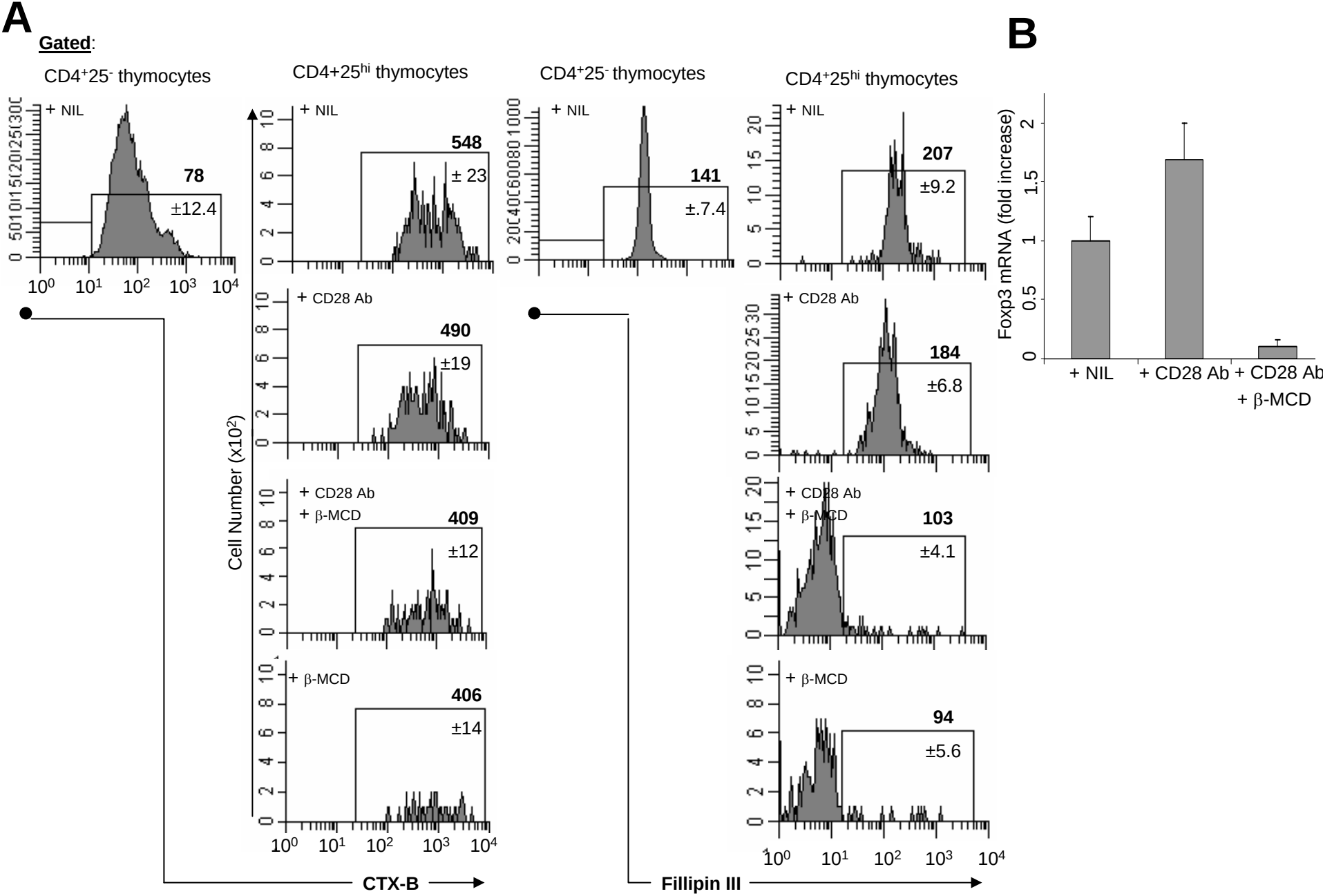

A
mRNA fold increase
c-myc
Foxp3
CD28 Ab
DRB (2h)
DRB (8h)
B
mRNA fold increase
CD28-CP mice
B10.D2 mice
+ LY294002
CD28 Ab
DRB (2h)

Figure 2


A
Gated:
CD4+25- thymocytes
CD4+25hi thymocytes
CD4+25- thymocytes
CD4+25hi thymocytes
+ NIL
78
±12.4
548
± 23
141
±.7.4
207
±9.2
+ CD28 Ab
490
±19
184
±6.8
+ CD28 Ab
+ β-MCD
409
±12
103
±4.1
+ β-MCD
406
±14
94
±5.6
Cell Number (x10²)
CTX-B
Fillipin III
B
Foxp3 mRNA (fold increase)
+ NIL
+ CD28 Ab
+ CD28 Ab
+ β-MCD

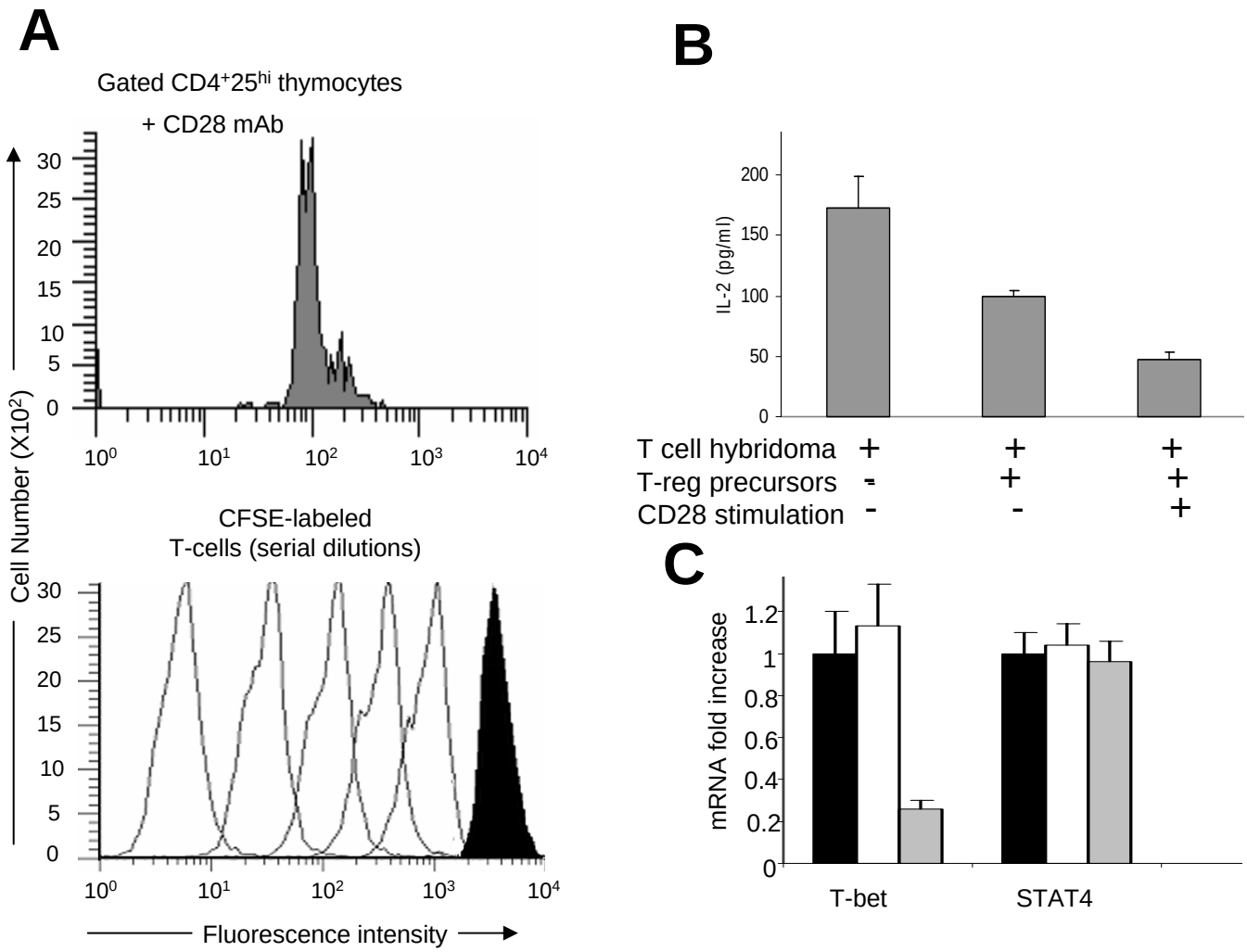

A
Gated CD4+25hi thymocytes
+ CD28 mAb
Cell Number (X10²)
30
25
20
15
10
5
0
10⁰
10¹
10²
10³
10⁴
CFSE-labeled
T-cells (serial dilutions)
Fluorescence intensity
B
IL-2 (pg/ml)
200
150
100
50
0
T cell hybridoma + + +
T-reg precursors - + +
CD28 stimulation - - +
C
mRNA fold increase
1.2
1
0.8
0.6
0.4
0.2
0
T-bet
STAT4

Figure 4

DISSERTATION DISCUSSIONS:

Although the molecular mechanisms of Treg cell development are still unclear, up-regulating the Foxp3 transcription factor is considered to be a crucial step in the thymic differentiation of these cells. Previous results showed that Foxp3 is detected in the CD4+8+ and CD4+8- stages of T cell development (Hori et al, 2003).

In the first part of this dissertation I found several new features of T reg development. Thus, we detected for the first time, Foxp3 transcript at the DN stage of T reg development by real time RT-PCR in two mouse strains sharing the same MHC class II (H2d haplotype). Supportive data to our findings obtained in mice were published very recently suggesting that human CD4-CD8- thymocytes express Foxp3. (Tuovinen et al, 2008). These data indicate that there is reason to believe that Foxp3 up-regulation precedes TCR expression. However, our results are more insightful than those presented by Tuovinen and colleagues, as we were able to define more precisely that Foxp3 is expressed at the DN3/4 but not DN1/2 stages of thymic development. The level of Foxp3 expression in thymus varied in different genetic backgrounds. We analyzed two different genetic backgrounds that share the same MHC class II haplotype and concluded that the differential expression of Foxp3 in these two genetic backgrounds was the results of a differential kinetics of T reg development.

I analyzed the potential mechanisms leading to a differential expression of Foxp3 in mice. Molecular mechanisms of epigenetic imprinting include selective demethylation of CpG motifs and histone modifications such as shown for cytokine genes (Ansel et al., 2003, Reiner, 2005, Tykocinski et al., 2005). Whether T reg cell differentiation also involves elements of epigenetic regulation has been unclear thus far. It has been reported

that an evolutionarily conserved region within the Foxp3 gene that contains CpG motifs, which are completely demethylated in regulatory T cells, but methylated in naïve and effector T cells. Another epigenetic chromatin modification, acetylation of histones, was also observed in T reg cells, and it was shown to be inversely correlated with methylation. (Floess et al., 2007). However, when we looked at epigenetic differences between the two strains, we noticed the same pattern of methylation of Foxp3 gene, suggesting that the differential expression of Foxp3 cannot be explained by difference in methylation in the sites studied. It remains to be investigated whether other epigenetic modifications such as acetylation, play a role, or if demethylation at different sites in the Foxp3 gene will provide more insights.

We observed a markedly different proliferation rate between the two mouse strains, with B10.D2 T reg cells proliferating at a higher rate. We also measured ObR on the surface of Treg precursors and found that it was expressed at much higher levels in T reg cells than in conventional T cells. Leptin is a cytokine-like hormone structurally similar to IL-6 and is involved in the control of food intake, metabolism, and T cell function (Friedman and Halaas, 1998; La Cava and Matarese, 2004). It has been reported that freshly isolated human Treg cells constitutively expressed high amounts of both leptin and the leptin receptor (ObR) and that the leptin pathway can act as a negative signal for the proliferation of Treg cells. These findings may partly explain why leptin-receptor deficiency associate with increased susceptibility to infection and resistance to autoimmunity (La Cava and Matarese, 2004; Ikejima et al., 2005; Farooqi et al., 2002; Matarese et al., 2002) and the increased risk of infection and reduced incidence of autoimmunity in individuals with low leptin (Matarese et al., 2002).

The T-reg cells represent only a fraction (5-10%) of the entire T-cell compartment. This may be explained by the negative signaling for proliferation mediated by ObR.

I also found a higher rate of apoptosis in T reg precursors from Balb/c mice, when compared with T reg precursors from B10.D2 mice. Also, specific apoptotic markers were found to be increased in Balb/c as compared with B10.D2. Thus, besides the differential rate of T reg thymic proliferation, the differential rate of thymic apoptosis is another mechanism by which the T reg compartment differs in the two strains.

Interestingly, the size of splenic $CD4^{+}25^{hi}$ T-reg compartment was found to be similar between the two mouse strains analyzed. This observation could be explained by the occurrence of an efficient homeostatic mechanism which operated efficiently to normalize the size of peripheral $CD4^{+}25^{hi}$ T-reg pool.

However, in contrast to the size of peripheral T-reg pool, the T-reg suppressogenic capacity was different in these two strains. As tested in a model of autoimmune diabetes, the splenic $CD4^{+}25^{hi}$ T-regs from B10.D2 mice were more suppressogenic and at the same time expressed more Foxp3 than their BALB/c counterparts. This is in agreement with previous studies that suggested that the suppressogenic capacity of T reg cells depends on the Foxp3 expression at the single cell level. Moreover, a recent study employing deletion of a conditional Foxp3 allele in mature peripheral T reg cells demonstrated that ablation of Foxp3 protein results in a loss of suppressor function and of phenotypic characteristics of T reg cells. Furthermore, these cells acquired the ability to produce IL-2 and pro-inflammatory cytokines and show pathogenic potential (Williams and Rudensky, 2007, Zheng and Rudensky, 2007). This is consistent with earlier findings that activated CD4+25- T cells with the highest amounts

of retrovirally expressed Foxp3 acquire suppressor function (Hori et al, 2003). These results indicate that epigenetic mechanisms and transcription factor networks set in motion by Foxp3 during T reg cell differentiation are unable to maintain lineage identity indefinitely. Instead, continuous expression of Foxp3 is needed to maintain the suppressogenic potential.

My findings revealed a fine balance between key regulators of thymic proliferation, differentiation, and selection in T-reg precursors such as CD3, ObR, Cdk4, Bad, and Caspase 3, which argues for a genetic and/or epigenetic control of T-reg development independently of the MHC class II-peptide presentation.

Molecular interactions of Foxp3 with other transcription factors are of great interest, particularly since the factor(s) responsible for inducing Foxp3 gene expression are poorly understood. It has been shown that Foxp3 regulates expression of different genes (IL-2, IFN-g) by association with other transcription factors such as NFAT (Wu et al., 2006), NF-kB (Bettelli et al., 2005), and Runx 1 (Ono et al., 2007). In addition, Smad3 and NFAT have been shown to cooperate to induce Foxp3 expression through its enhancer (Tone et al., 2008). Description of Foxp3 – dependent functional and transcriptional programs will provide new information on the role of Foxp3 as a specification factor for T reg differentiation and suppressor function.

In the second part of my study, I provide insights into the role CD28 plays in T reg biology. It was shown that superagonistic anti-CD28 antibodies are potent activators of T reg cells (Beyersdorf et al, 2005). Here, I showed that stimulation of T reg cells in vitro with an anti-CD28 Ab leads to significant up-regulation of Foxp3 gene expression, this being correlated with the observation that CD28 -/- mice express very low levels of

Foxp3. Stimulation with anti-CD28 Ab could not induce de novo transcription of Foxp3 in DN1 and DN2 stages of T cell differentiation, which clearly indicated that the CD28 pathway cannot induce de novo expression of Foxp3 in the thymus.

Next I compared the span life of Foxp3 transcript in the absence or presence of CD28 costimulation. By using a chemical reagent which inhibits RNA polimerase II and therefore stops transcription, we were able to show that stimulation with anti-CD28 Ab stabilizes the Foxp3 transcript, and prolongs its half life up to a period of 8 h. This is especially important, since it was previously shown that CD28 costimulation is able to stabilize mRNA expression of several cytokines in conventional T cells (Fraser et al, 1991, Pioli et al, 1998). CD28 is known to activate several signaling pathways in T cells (Ledbetter et al, 1990). We examined whether the Foxp3 mRNA stabilization by CD28 is dependent on its PI3K signaling pathway or p56 Lck signaling pathway. According to my findings, a model of signaling to Foxp3 expression in T reg precursors is presented

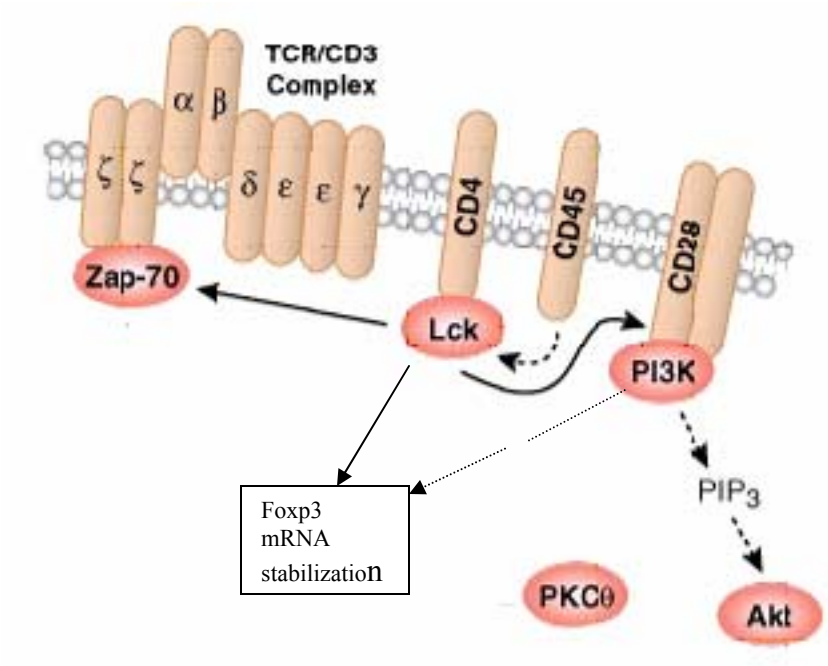


Figure IV: Signaling pathways in T cells.

Using a mouse KO for p56 Lck binding site on CD28 cytoplasmic tail, I clearly showed that CD28 stabilizes Foxp3 mRNA through the p56 Lck binding motif, and is independent on its activation of PI3K signaling pathway.

Since our experiments indicated that CD28 costimulation provides positive signaling for Foxp3 expression by a mRNA stabilization mechanism, we addressed the role of lipid rafts in this process (Figure V).

Lipid rafts are known to represent a signaling platform for conventional T cells. Involvement of lipid rafts in multiple signaling reactions at the immunological synapse has been extensively studied. Lck, Fyn, and LAT constitutively associate with raft domains *via* lipid-modification (Webb et al., 2000). Targeting of these signaling proteins into nonraft membranes results in an impaired T-cell activation (Zhang et al., 1998, Kabouridis et al., 1997). ZAP70, PLC-g, Vav1, PKC-y, and Wiskott– Aldrich syndrome protein (WASP) are also shown to be recruited to lipid rafts upon TCR triggering (Johmura et al., 2003, Bi et al., 2001, Dupre et al., 2002). Depletion of cholesterol with methyl-b-CD (MβCD) disrupts lipid rafts, which inhibits TCR-induced calcium mobilization (Rouquette-Jazdanian et al., 2002). Altogether, these results suggest the essential roles of lipid rafts in T-cell signaling. Recently it has been shown that ligation of CD28 by its natural ligand CD86 alone can induce lipid rafts polarization. This suggests that lipid rafts polarization in primary human CD4+ T cells is not only driven by a costimulatory signal, it can also be independent of a TCR signal (Kovacs et al., 2005).

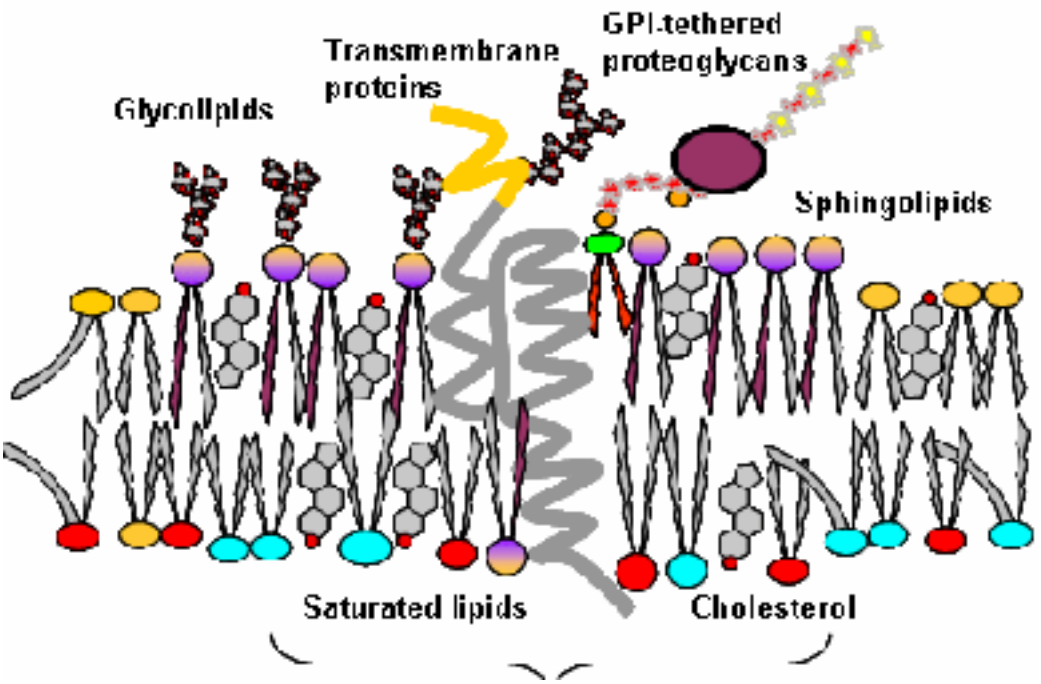


Figure V: Structure of lipid rafts on the plasma membrane

I found a significant difference between lipid raft partitioning in T reg cells and conventional T cells. The GM1 glycosphingolipids and cholesterol were expressed at a higher level in T reg cells. Moreover, there was no significant change in the lipid rafts partitioning after CD28 costimulation.

However, lipid rafts were drastically affected by a cholesterol depleting agent (MβCD). When providing CD28 costimulation to thymic T reg cells with an anti-CD28 Ab, the T reg cells pretreated with MβCD failed to show any Foxp3 gene expression up-regulation, suggesting that lipid rafts are essential elements in stimulation of T reg cells by CD28.

It remains to be investigated which is the mechanism by which lipid rafts may be involved in the function of T reg cells.

When I examined T reg cell function in vitro, I noticed a significant increase in their suppressogenic capacity after stimulation with anti-CD28 Ab. We used a novel in vitro functional assay set up in our lab, in which hybridoma cells represent the responder cells to T reg suppression. This is an advantage because it is well known that hybridoma cells do not require co-stimulation for growth and cytokine secretion; therefore they represent a reliable and easy to work T responder cell population. Overall, I provided evidence for a new mechanism by which CD28 costimulation solely up-regulates Foxp3 expression in T reg thymic precursors through stabilization of mRNA transcript. CD28-mediated PI3K activation was not essential for this activity, but the p56 Lck binding motif on the cytosolic CD28 tail was proven to be critical. Also, I showed that lipid rafts integrity is essential for CD28 signaling events leading to up-regulation of Foxp3 expression in thymic T reg precursors.

Accumulating evidence suggests that the immunosuppressive potential of T reg cells can be used as a therapeutic strategy in autoimmune diseases, infections and transplantation, as well as tumor immunotherapy. A better understanding of T reg cells biology will make soon use of their potential therapeutic effects in a variety of diseases.

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Apendix

Statement of Author's Contribution:

The author was personally involved in the planning and execution of all experiments described in this dissertation, and played a crucial role in the writing of both papers presented here. The author also developed the in vivo CFSE labeling of cells, in vitro suppressogenicity assay using a hybridoma cell line and was directly involved in additional experimental assays used for these studies. Furthermore, the author has presented different stages of this work in poster presentations at a number of scientific meetings, in addition to coauthoring papers not directly related to this dissertation. This dissertation serves as partial fulfillment of the dissertation requirements for the Molecular and Cell Biology program of USUHS, Bethesda, MD 20814.